REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	11 December 1992	Final report, 11 December 1992

4. TITLE AND SUBTITLE
Studies on the Biodegradation of Ordnance-Related Hazardous Waste, Phase I.

5. FUNDING NUMBERS
N/A

6. AUTHOR(S)
Rakesh Govind

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Chemical Engineering
Mail Location 171
University of Cincinnati
Cincinnati, OH 45221

8. PERFORMING ORGANIZATION REPORT NUMBER
N/A

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
SERDP
901 North Stuart St. Suite 303
Arlington, VA 22203

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
N/A

11. SUPPLEMENTARY NOTES
Submitted to Naval Ordnance Station, Indian Head, MD, 11 December 1992.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release: distribution is unlimited

12b. DISTRIBUTION CODE
A

13. ABSTRACT ***(Maximum 200 Words)***

Bioprocessing or biodegradation often provides a low-technology, permanent, inexpensive, effective, nonpolluting alternative for land reclamation and treatment of industrial effluents. Microbes have evolved or can be adapted to degrade virtually any toxic organic chemical. Hydrocarbons, a major class of military wastes, are particularly susceptible to biodegradation. Biodegradation is effective over a range of environmental conditions, and for a wide variety of contaminants. Often, bioprocesses can be integrated with conventional technologies, resulting in efficient, multicomponent systems.

14. SUBJECT TERMS
bioprocessing, biodegradation, hydrocarbon, SERDP

15. NUMBER OF PAGES
28

16. PRICE CODE
N/A

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
unclass	unclass	unclass	UL

BIODEGRADATION OF NITRATE ESTERS SERDP 91+92

7 - 1992

FINAL REPORT

on

STUDIES ON THE BIODEGRADATION OF ORDANCE-RELATED HAZARDOUS WASTE

PHASE I

CATEGORY: MANUFACTURING OF ORDANCE AND ORDANCE COMPONENTS AND CHEMICALS

SUBMITTED TO: JOHN STACEY
Code 2810C
Naval Ordnance Station
Indian Head, MD 20640-5000

SUBMITTED BY: Dr. Rakesh Govind
Department of Chemical Engineering
Mail Location 171
University of Cincinnati
Cincinnati, OH 45221

December 11, 1992

TABLE OF CONTENTS

Introduction 1

Project Objective 2

Background 2

Experimental Studies on Aerobic Degradation 4

- Development of acclimated master cultures 4
- Use of electrolytic respirometry for aerobic kinetics 5
- Results and Discussion 7

Experimental Studies on Anaerobic Degradation 10

- Establishment of Master Culture Reactors 10
- Determination of Anaerobic Kinetics 10
- Preparation of chemical stock solution and analytical methods 11
- Results and Discussion 12

Conclusions 14

Recommendations for future work 15

References 16

List of Tables

List of Figures

INTRODUCTION

The molecular processes involved in microbial and enzymatic degradation are being exploited by agencies of the U.S. Department of Defense in diverse ways. Biodegradation often provides an attractive or adjunct to conventional methods of restoring contaminated soils or groundwater, or treating wastewater containing hazardous chemicals. In this report, biodegradation refers to the use of microorganisms or enzymes to break down chemical compounds.

In 1980, Congress enacted legislation to identify, finance, and monitor cleanup of the nation's most serious hazardous waste dumps. Currently, DOD has 739 sites in need of remediation, and cleanup costs are estimated at five to 10 billion U.S. dollars. The primary hazardous wastes found on military installations are fuels, cleaning solvents, propellants, explosives, etc. Specifically, this proposal is addressing ordnance-related hazardous chemicals, such as Nitroglycerine, Glycerol trinitrate, Propylene Glycol Dinitrate, Triethylene glycol dinitrate, and Tri methyl ethane trinitrate.

Virtually all conventional technologies for land reclamation have proved to be unsatisfactory. For example, landfilling is not a permanent solution, and costs are increasing dramatically (in the United States, from US $10 to US $250 per ton in the last five years). Incineration produces toxic air pollutants, and many organic compounds are difficult to burn. In situ solidification (chemical fixation), capping, and vitrification are largely unproved, costly, and aesthetically objectionable technologies. Volatile organics in groundwater have been removed via strategically located slotted wells and by air stripping where contaminated groundwater is pumped through large packed-bed towers; both are costly, labor-intensive solutions and require further treatment of the contaminated air stream that is generated.

Furthermore, conventional methods for treatment of wastewater are expensive and require further treatment of the gas and solid phases that are generated. For example, conventional activated sludge treatment requires large-scale plant size, with a retention time in the range of 6-9 days, and generates contaminated air phase and sludges requiring further treatment.

Bioprocessing or biodegradation often provides a low-technology, permanent, inexpensive, effective, nonpolluting alternative for land reclamation and treatment of industrial effluents [1]. Microbes have evolved or can be adapted to degrade virtually any toxic organic chemical. Hydrocarbons, a major class of military wastes, are particularly susceptible to biodegradation. Biodegradation is effective over a range of environmental conditions, and for a wide variety of contaminants. Often, bioprocesses can be integrated with conventional technologies, resulting in efficient, multicomponent systems.

PROJECT OBJECTIVE

The specific objective of this project was to determine aerobic and anaerobic biodegradation kinetics for the following compounds: Nitroglycerine, Propylene Glycol dinitrate (PGDN), Triethylene glycol dinitrate (TEGDN) and Trimethyloethane trinitrate (TMETN). Electrolytic respirometers were used for determining aerobic kinetics. Biological methane production and disappearance of parent compound were used for determining anaerobic kinetics.

BACKGROUND

The microbial degradation of glycerol trinitrate (GTN) has been reported in the literature [2]. The degradation process proceeds through a series of successive denitration steps through glycerol dinitrate and glycerol mononitrate isomers, with each succeeding step proceeding at a slower rate.

In addition to direct microbial degradation, chemical treatments of GTN have been developed in order to desensitize waste streams, resulting in the disappearance of glycerol tri-, di-, and mononitrates, but with the corresponding formation of glycidol and glycidyl nitrate [3]. These products contain a highly reactive epoxide moiety that tends to confer mutagenic properties.

The chemical by-products, glycidol and glycidyl nitrate, have also been studied to determine their biodegradability. The pathway from glycidyl nitrate to glycerol 1-mononitrate to glycerol proceeds more slowly with each succeeding step. The steps from glycidol to glycerol and glycidyl nitrate to glycerol 1-mononitrate occur spontaneously in aqueous solutions but appear to be accelerated (directly or indirectly through secondary effects) by microbial activity [3].

Glycidol and glycidyl nitrate tested positive in the Ames test screening for mutagenicity, whereas the transformation product glycerol-1-mononitrate tested negative [3]. Both glycerol trinitrate and glycerol-1-mononitrate have been shown to be toxic to mammals [4].

Propylene glycol dinitrate (PGDN), diethylene glycol dinitrate (DEGN), triethylene glycol dinitrate (TEGDN), and trimethyllolethane trinitrate (TMETN) undergo microbial transformation via successive denitration steps, leading to the formation of the corresponding glycols:propylene glycol (PG), diethylene glycol (DEG), triethylene glycol (TEG), and trimethylolethane glycol (TMEG) (refer Figure 3)[5].

The degradation of the resulting glycols at concentrations of 100 mg per liter has also been assessed [6]. Rates of degradation were as follows: PG > DEG > TEG > TMEG, from high to low, although degradation appeared to be due to a combination of biological and non-biological processes.

PG, DEG, and TEG present minimal toxicity and carcinogenic hazards. PG is the least toxic of the glycols and is commonly used in pharmaceutical, cosmetic, and food applications. DEG and TEG are slightly toxic; repeated large doses are needed for toxicity [7]. TMEG had negative test results in AMES test screening for mutagenicity [6].

A generic biological treatment concept for wastewaters contaminated with hazardous Nitrate Esters is as follows:

```
INFLUENT  ----> CHEMICAL PRETREATMENT ----->
                  (if required)

ANAEROBIC
DENITRIFICATION -----> AEROBIC TREATMENT ---> EFFLUENT
```

Mixed cultures were used in many of the studies reported here. The ability of microorganisms to biotransform this class of compounds is apparently ubiquitous in nature. The use of acclimatized cultures from environments previously exposed to the compounds under study perhaps would have accelerated some of the initial rates of transformation observed; however, once acclimatized, random environmental microbial inocula were capable of transforming the compounds studied.

EXPERIMENTAL STUDIES ON AEROBIC DEGRADATION

Development of acclimated master cultures:

Master cultures were developed for three organic compounds: Propylene Glycol Dinitrate, Trimethyloethane Trinitrate, and Nitroglycerine, by exposing wastewaters containing these compounds to a domestic wastewater treatment plant culture. A batch stirred tank reactor, 5 liters in volume, was operated at 10 C to prevent growth of nitrifying bacteria, with continuous aeration of air. To start the reactor, activated sludge and sewage from the Miami

Municipal Treatment Plant in Cincinnati, Ohio, was centrifuged and then allowed to settle in settlers. The supernatant was filtered through a #1 Whatman filter to remove protozoa, and then filled into the batch stirred tank master culture reactor. Wastewaters, supplied by the Navy, were diluted with distilled water (1:8 ratio), and then supplied to the master culture reactor. The feed to the reactor (200 ml fed daily) consisted of an equimolar mixture of PGDN, TMETN, and GTN diluted wastewaters. 200 ml of OECD composition nutrients were also fed daily. 400 ml of mixed liquor was withdrawn daily from the reactor, to maintain the volume of liquid in the reactor.

The reactor was monitored daily for nitrates and nitrites.

Use of Electrolytic Respirometry for Aerobic Kinetics:

Electrolytic Respirometry studies were conducted using an automated continuous oxygen measuring Voith Sapromat B-12 (Voith-Morden, Milwaukee, WI). The system consists of a temperature controlled bath which contains the measuring units, a recorder for digital indication, a plotter for direct presentation of the oxygen uptake curves of substrates, and a cooling unit for conditioning and continuous recirculation of water bath contents. The system used has 12 measuring units each connected to a recorder. Each unit, consists of a reaction vessel, with a carbon dioxide absorber (soda lime) mounted in a stopper, an oxygen generator, and a pressure indicator. The unit is sealed from the atmosphere, and hence fluctuations in barometric pressure does not affect the results. The reactor contents are well stirred by a magnetic stir bar, thus ensuring effective gas exchange. Biodegradation of the organic contents in the reactor vessel, creates carbon dioxide, which is absorbed by the soda lime pellets, thereby creating a negative pressure in the flask. This decrease in reactor vessel pressure triggers the pressure sensor which switches the oxygen

generator. Oxygen is generated by electrolysis of a copper sulfate -sulfuric acid solution. The oxygen flows into the reactor vessel, thereby restoring the oxygen content in the headspace. The amount of oxygen generated is monitored by measurement of the voltage supplied to the electrolytic unit, and the cumulative oxygen generated per unit volume of the reactor flask contents, is plotted as the oxygen uptake curve.

The nutrient solution is made as per Organization of Economic Cooperation and Development (OECD) guidelines. It contains 10 ml of solution A and 1 ml of each of the solutions B to F per liter of synthetic medium : solution A - KH_2PO_4 8.5 g, K_2HPO_4 21.75 g, $Na_2HPO_4.2H_2O$ 33.4 g, and NH_4Cl 2.5 g; solution B - $MgSO_4.7H_2O$ 22.5 g; solution C - $CaCl_2$ 27.5 g; solution D - $FeCl_3.6H_2O$ 0.25 g; solution E - $MnSO_4.4H_2O$ 39.9 mg, H_3BO_3 57.2 mg, $ZnSO_4.7H_2O$ 42.8 mg, $(NH_4)_6Mo_7O_{24}$ 34.7 mg and $FeCl_3$.EDTA 100 mg; and solution F - Yeast extract 150 mg. The chemicals for each of the solutions A, B, C, D, E and F, are dissolved in 1000 ml of deionized water. The solution D is freshly prepared immediately before the start of an experiment.

The microbial inoculum was obtained from the acclimated culture reactor by centrifuging the mixed liquor. The dry weight of biomass was measured by drying samples, in duplicates, of 1 ml, 2 ml, and 3 ml at 105 C overnight. A concentration of 30 mg/l of sludge as dry weight was used in each reactor vessel. The total volume of the synthetic medium in the 500 ml capacity reactor vessels was brought up to a final volume of 250 ml.

The concentration of the test compound was varied. Aniline was used as the biodegradable reference compound. The design of the experimental runs is shown in Table 1.

Table 1: Experimental Design of the Aerobic Respirometer Runs

RESPIROMETER NUMBER --

CHEMICAL NAME: _____

Flask No.	Inoculum	Chemical	Nutrient	Water
1	8 ml	25 ml	167 ml	50 ml
2	8 ml	25 ml	167 ml	50
3	8 ml	25 ml	167 ml	50
4	8 ml	50 ml	167 ml	25
5	8	50	167	25
6	8	50	167	25
7	8	75	167	0
8	8	75	167	0
9	8	75	167	0
10	8	25+5A	167	0
11	8	15+5A	167	0
12	8+2 ml HgCl2	50	167	23

Note that the chemical was added in amounts of 25 ml, 50 ml and 75 ml of 1000 ppm stock solution. The nutrients was basic OECD synthetic medium. Deionized distilled water was used to make up the flask contents to 250 ml. In flasks 10 and duplicate 11, a solution of aniline (5 m/l concentration), which is the reference compound, was used as a toxicity control. In flask 12, 2 ml of a mercuric chloride (200 ppm concentration) was added, so that no bacterial action occurs.

Results and Discussion

The oxygen uptake results are shown in Figures 1 through 4 for Nitroglycerine, Propylene Glycol Dinitrate and Trimethyloethane Trinitrate compounds, respectively. Note that Propylene glycol Dinitrate (PGDN) and Trimethyloethane Trinitrate (TMETN) were not biodegraded at all in the respirometer. Nitroglycerine was degraded in the respirometer, but there was a long acclimation period of 24 hours (refer Figure 1) before

glycerol was formed. The glycerol product was easily biodegraded. Aniline degradation in the presence of nitroglycerine (Figure 2) showed delayed onset of aniline degradation and incomplete mineralization of the aniline. In Figure 2, the first plateau of oxygen uptake is attained due to aniline degradation, followed by degradation of nitroglycerine.

Figure 3 shows the oxygen uptake curve for PGDN solutions. Note that no substantial uptake of oxygen occurred, even when aniline was present in the solution. This shows that PGDN was not degraded aerobically and that it also inhibited the degradation of aniline. Similar results were obtained for TMETN, as shown in Figure 4.

No attempts were made to quantify the aerobic biodegradation kinetics, since the compounds were either very slow to acclimate or did not biodegrade at all, at the three concentrations, used in the respirometer. Clearly, aerobic biodegradation conditions were not optimal for biotransformation of these compounds.

Aerobic studies were conducted with NG concentration of 100 ppm and PGDN, TEGDN and TMETN concentrations of 20 ppm each. Figure 5 shows oxygen uptake curve for 100 ppm NG water when mixed with 20 ppm each of PGDN and TMETN. With 20 ppm each of PGDN and TMETN, the extent of NG degradation was decreased, as noted by the oxygen uptake curve. The total oxygen demand gave a plateau at 80 mg/L of oxygen, when the theoretical oxygen demand should have been 120 mg/L. This indicated that 20 ppm of PGDN and TMETN was toxic to the aerobic culture.

Two cultures obtained from Dr. Marilyn K. Speedie, University of Maryland at Baltimore, were cultured in mixed aerobic master culture reactors. Samples of the culture were taken from these reactors and used in the electrolytic respirometric studies for the degradation of NG. There was an

initial oxygen demand which then rapidly decreased after a short time period of 2 hours, indicating the generation of a toxic intermediate which strongly inhibited aerobic degradation.

EXPERIMENTAL STUDIES ON ANAEROBIC DEGRADATION

Establishment of Master Culture Reactors:

Two master culture reactors, shown in Figure 6, consisting of 13 liter capacity pyrex glass vessels, were started using a seed culture obtained from an anaerobic reactor operating at a treatment plant facility. The seed culture was screened to remove sediment and large floc and then resuspended in the master culture reactors. The master culture reactors were operated under the following conditions:

1. Temperature = 35°C
2. Hydraulic and Solids retention time: 20 days
3. A draw-and-fill schedule
4. A COD loading rate of 1.0 g/L-d, and
5. A constant nutrient/mineral/buffer medium.

The operating procedure of the master culture reactor is given in Table 2. The composition of the nutrient media is given in Table 3.

A Varian 3700 gas chromatograph with an FID detector was used to measure the concentration of volatile acids. Standard calibration curves for acetic acid and propionic acid are shown in Figures 7 and 8, respectively.

Determination of Anaerobic Kinetics:

A standard batch anaerobic bioassay technique (Biological Methane Potential (BMP) and Anaerobic Toxicity Assay (ATA)) was used to determine the methane potential and the anaerobic toxicity of Nitroglycerine, TMETN, TEGDN and PGDN.

The BMP assay was conducted with Corning no. 1460, 250 ml reagent bottle and ATA assay was conducted with 125 ml reagent bottles with rubber septum caps of appropriate size. The bottles were gassed at a flow rate of approximately 0.5 l/min. with 30% CO2 and 70% N2, then stoppered and equilibrated at incubation

temperature of 35 C prior to introducing samples, defined media and inoculum. The defined media contained nutrients and vitamins for mixed anaerobic cultures. Resazurin was added to detect oxygen contamination and sodium sulfate was added to provide a reducing environment. The final assay concentrations of nitrogen, phosphorus, and alkalinity were respectively: 12 mg/l as N, 19 mg/l as P and 2500 mg/l as CaCO3.

For BMP assay, inoculation was accomplished anaerobically by inserting a gas flushing needle into the neck of the media flask while adding 200 ml of seed organisms to 1800 ml of defined media. A 20% by volume inoculum was used.

Samples were added to the serum bottles anaerobically, and experiments at concentrations of 100 ppm, 500 ppm, 1000 ppm, 2000 ppm were run in triplicate. A seed blank with no addition of chemical was also run in triplicate.

For ATA assay, a "spike" containing acetate and propionate in addition to the seed blanks was also added to each bottle. A control with only the spike was also prepared. Each bottle contained 2 ml of acetate-propionate solution containing 75 mg acetate and 26.5 mg propionate.

Gas volume production was measured with the help of a calibrated pressure transducer. The composition of the gas was also measured using a Gas Chromatograph.

Studies were also conducted by filtering a sample of the liquid from the serum bottle and analyzing the concentration of the parent compound using HPLC analysis.

Preparation of Chemical Stock Solution and analytical methods:

The water sample, sent by the Navy, was extracted with ethyl ether in a continuous extractor for 24 hours, which very efficiently extracted essentially all the nitrate ester

compounds. The extracts were concentrated by evaporation of solvent, at room temperature. Quantitative analysis of the nitrate ester compound was made by using a Hewlett Packard GC/MS and a Waters HPLC in conjunction with a silica column (Varian Micropak Si, 10 um) 50 cm in length with 2.2 mm ID. Analysis was conducted at 196 nm with a back pressure of 1,800 lb/sq. in. The HPLC method is summarized in Table 4 and is derived from a NG analytical method developed by SAIC, Inc. (attached in Appendix 1). Figure 9 shows a standard analysis of the four nitrate ester compounds using a flowrate of 3.0 ml/min.

The analyses were calibrated using the standards, sent by the Navy.

Results and Discussion

Anaerobic Serum Bottle Results

The anaerobic serum bottle results (BMP) are shown in Figures 10 through 12, for Nitroglycerine, PGDN and TMETN, respectively. Each curve was obtained as the average of the triplicate runs. The concentration of the compounds were 100 ppm, 500 ppm, 1000 ppm, and 2000 ppm.

All three compounds degraded anaerobically at all concentrations. Nitroglycerine degraded very rapidly and showed no toxicity to the anaerobes. PGDN degraded slower than nitroglycerine, and note that the gas production at 2000 ppm was lower than at 1000 ppm, indicating some toxicity to the anaerobic culture. TMETN degradation kinetics was slower than PGDN and also exhibited toxicity to the culture at 2000 ppm.

Clearly, anaerobic degradation would be very successful in degrading these chemical compounds. However, it should be mentioned, that maintenance of anaerobic conditions, in actual bioreactors is difficult, and would require very careful design for successful operation.

Attempts to obtain the upper limiting concentration for PGDN and TMETN when toxicity is created for the anaerobic culture were unsuccessful, due to gas volume measurement errors.

The BMP results were analyzed using a standard Haldane kinetics model, and the resulting kinetic constants are given in Table 5. These values of the kinetic constants would be useful in the design of the biofilter system for biological treatment of wastewaters containing these compounds.

Figure 13 shows the gas production with time for the mixture of NG (500 ppm), PGDN (500 ppm) and TMETN (500 ppm). It can be seen that the mixture degraded very easily and cumulative methane production corresponded to the data obtained for the individual compounds.

Figure 14 shows the cumulative methane production for TMETN water at a concentration of 1500 ppm. The cumulative gas production was higher than that for 1000 ppm indicating that at this concentration, TMETN had no toxicity effects to the culture. Hence it can be concluded that toxicity effects for TMETN occur between 1500 ppm and 2000 ppm.

Concentration of the parent compounds were also measured as a function of time in the serum bottles for two initial concentration sof NG of 50 mg/L and 100 mg/L. Figure 15 shows the concentration of NG decreasing with time for an initial concentration of 50 mg/L and 100 mg/L and 10 mg/L with ethanol. The abiotic control is shown in this figure for comparison.

Figure 16 shows the response peak area versus time for NG at an initial concentration of 50 mg/L. Clearly NG concentration decreases with time. The intermediate shown at a retention time of 3.42, which corresponds to Glycerol dinitrate, increases initially and then decreases due to degradation. Glycerol mononitrate, shown at a retention time of 1.5 increases initially

and then eventually declines. Glycerol, corresponding to a retention time of 0.97 increases and then remains at a steady value. Similar data was obtained for NG at an initial concentration of 100 mg/L, as shown in Figure 17.

This data shows that the pathway for the degradation of NG is as follows:

Glycerol trinitrate (NG) ----> Glycerol dinitrate ----> ----> Glycerol mononitrate ----> Glycerol

Studies were also conducted for PGDN and the kinetic data is shown in Figure 18. The concentration of PGDN declines in each experiment conducted at initial PGDN concentrations of 10, 50 and 100 mg/L. Similar data for TMETN and TEGDN are being obtained and the results will be reported later in an addendum to this report.

CONCLUSIONS

NG degrades under aerobic conditions at an initial concentration of 100 ppm after a long acclimation time of 24 hours. PGDN, TMETN And TEGDN did not degrade aerobically.

NG, PGDN, TMETN and TEGDN degraded anaerobically very easily and at concentrations as high as 1000 ppm. The gas production data was analyzed to obtain Haldane kinetic parameters for the nitrate ester compounds.

Detailed kinetic studies using measurement of the parent compound showed that NG degrades by sequential release of the NO_2 group.

RECOMMENDATIONS FOR FUTURE WORK

Results reported here show that anaerobic treatment of wastewater containing nitrate ester compounds is feasible. Further studies need to be conducted with an actual anaerobic reactor system using an immobilized biofilm system to demonstrate the economics of using anaerobic treatment for wastewaters containing NG, TMETN, TEGDN and PGDN.

REFERENCES

[1] K. Gold and B.W. Brodman. U.S. Patent No. 4,756832 (july 12, 1988).

[2] T.M. Wendt, J.H. Cornell, and A.M. Kaplan, Applied Environ. Microbiol., 36, 693 (1978).

[3] D.L. Kaplan, J.H. Cornell, and A.M. Kaplan, Applied Environ. Microbiol., 43 (1), 144 (1982).

[4] L.V. Melinikoveend, A.M. Klyachkina, Toksikol Nov. Prom. Khima Veshchestv, 15, 97 (1979).

[5] J.H. Cornell et al., Report No. 81.029, U.S. Army Natick Research, Development and Engineering Center, Natick, MA 1981.

[6] D.L. Kaplan, J.T. Walsh, and A.M. Kaplan, Environ. SCi. Tech. 16 (10), 723 (1982).

[7] R.E. Gosselin et al., Chemical Toxicity of Commercial Products, 4th Edition (Williams and Wilhem, Baltimore, MD 1976).

LIST OF TABLES

Table 1. Experimental design of the aerobic respirometer runs.

Table 2. Operating procedure for the master culture reactors.

Table 3. Composition of the nutrient media fed to the anaerobic master culture reactors.

Table 4. Summary of the HPLC analytical method for analyzing nitrate esters.

Table 5. Kinetic parameters for the anaerobic degradation of nitrate ester compounds.

Table 2. Operating procedure for the master culture reactors.

Start-Up

1. A starting seed culture was added to a 13-liter glass vessel. The seed culture was obtained from an operational anaerobic digester and diluted with distilled water to provide a starting solids concentrations of about 2,000 mg/L. Mixing was accomplished by using a magnetic stirring unit.

2. A feed stock consisting of 20,000 mg/L ethanol in Nutrient/Mineral/Buffer medium was fed to the Master Culture Reactor daily to produce a 20-day hydraulic retention time (600 mL to a 12-L culture volume).

3. The Master Culture Reactor was operated continuously for more than 60 days to allow steady-state conditions to be established.

Daily Operation

1. The gas volume produced in the reactor was measured daily by using an automatic anaerobic respirometer.

2. A fixed volume equal to 5 % of the mixed liquor was removed daily to provide a 20-day hydraulic and solids retention time. The connecting lines were flushed prior to each feeding with a 70 % N_2/30 % CO_2 mixture to avoid drawing air into the unit.

3. The pH was measured immediately after withdrawal; VSS and volatile acid residuals were measured on a weekly basis.

4. 600 mL of substrate, prepared by adding a suitable amount of acetate or ethanol to Nutrient/Mineral/Buffer solution was added to the reactor. When the MCR was less than full, the substrate was added in amounts equal to 5 % of the volume in inventory until the reactor was again full.

Table 3. Composition of the nutrient media fed to the anaerobic master culture reactors.

1. Mineral Base I

   a. Add the following to 600 mL of distilled water

$CoCl_2.6H_2O$	0.25 g	$NaMoO_4.2H_2O$	0.005 g
$FeCl_2.4H_2O$	2.0 g	$NiCl_2.6H_2O$	0.025 g
$MnCl_2.4H_2O$	0.05 g	Na_2SeO_4	0.025 g
H_3BO_3	0.025 g	$ZnCl_2$	0.025 g
$CuCl_2$	0.015 g		

   b. Dilute to 1.0 liter (Note: this mixture may form a light precipitate and should be agitated vigorously before transferring)

2. Mineral Base II

   a. Add the following to 600 mL distilled water

$CaCl_2$	38[a] g
$MgCl_2.6H_2O$	50[b] g

   b. Dilute to 1.0 liter

3. Nutrient Base

   a. Add the following to 600 mL distilled water

KH_2PO_4	135[c] g
K_2HPO_4	175[d] g
NH_4Cl	53 g
Na_2SO_4	15 g

   b. Dilute to 1.0 liter

4. Buffer Base

   a. Add the following to 600 mL distilled water

$NaHCO_3$	60 g

   b. Dilute to 1.0 liter

Table 4. Summary of the HPLC analytical method for analyzing nitrate esters.

METHOD (from Baaske et al JNL PHARM SCI 72,2, FEB 83)

COLUMN: uBONDAPAK PHENYL 8X10 , 10uM

SOLVENT: 64% WATER/36% ACETONITRILE

FLOW: 1.50 ml/ min

DETECTION:WATERS PHOTODIODE ARRAY(190-350nm scan)

COMPOUND of INTEREST: Trimethyloleethane Trinitrite (TMETN), Glycerol Trinitrite (GTN), Propyleneglycol Dinitrate (PGDN), Triethylene glycol Dinitrate (TEGDN).

ALTERNATIVE HPLC METHOD:

COLUMN: SAME

SOLVENT: SAME

DETECTION: Waters M481 Variable Wavelength Detector monitoring at 196nm/0.2AUFS.

FLOW: 1.50 ml min^{-1} using a Waters M45 pump with a Waters U6K injector.

	A	B	C	D
1	COMPOUND	QUANT(nm)	% PURITY	LOD (ug)
2	TEGDN	196	95	0.08
3	PGDN	193	96	0.04
4	GTN	193	95	0.04
5	TMETN	193	97	0.35

Table 5. Kinetic parameters for the anaerobic degradation of nitrate ester compounds.

Toxicant	Concentration (ppm)	k (mg/mg VSS-hr)	Ks (mg/l)
Nitro-Glycerine	100	0.26	45
	500	0.25	48
	1000	0.27	47
	2000	0.26	50
PGDN	100	0.15	82
	500	0.14	85
	1000	0.16	80
	2000	0.08	95
TMETN	100	0.10	95
	500	0.09	100
	1000	0.08	102
	2000	0.04	110

LIST OF FIGURES

Figure 1. Oxygen uptake curves for aerobic degradation of nitroglycerine.

Figure 2. Oxygen uptake curves for aerobic degradation of nitroglycerine with aniline.

Figure 3. Oxygen uptake curves for PGDN solution.

Figure 4. Oxygen uptake curves for TMETN solution.

Figure 5. Oxygen uptake curves for aerobic degradation of nitroglycerine (NG) and mixture of NG with PGDN and TMETN.

Figure 6. Schematic of the master culture reactor.

Figure 7. Standard calibration curve for analysis of acetic acid in the anaerobic master culture reactor.

Figure 8. Standard calibration curve for analysis of propionic acid in the anaerobic master culture reactor.

Figure 9. Standard curves obtained using HPLC analysis for nitrate ester compounds.

Figure 10. Anaerobic serum bottle results for nitroglycerine.

Figure 11. Anaerobic serum bottle results for PGDN.

Figure 12. Anaerobic serum bottle results for TMETN.

Figure 13. Gas production data for a mixture of NG, PGDN and TMETN.

Figure 14. Cumulative methane production for TMETN water at a concentration of 1500 ppm.

LIST OF FIGURES (continued)

Figure 15. Changes in the concentration of NG with time in the anaerobic serum bottle.

Figure 16. Response peak area versus time for NG at an initial concentration of 50 mg/L.

Figure 17. Response peak area versus time for NG at an initial concentration of 100 mg/L.

Figure 18. Kinetic data for PGDN solution.

FIGURE 1: Oxygen Uptake curves for aerobic degradation of Nitroglycerine (Flasks 1,2,3 in Table 1). Th lowest curve is the mercuric chloride control.

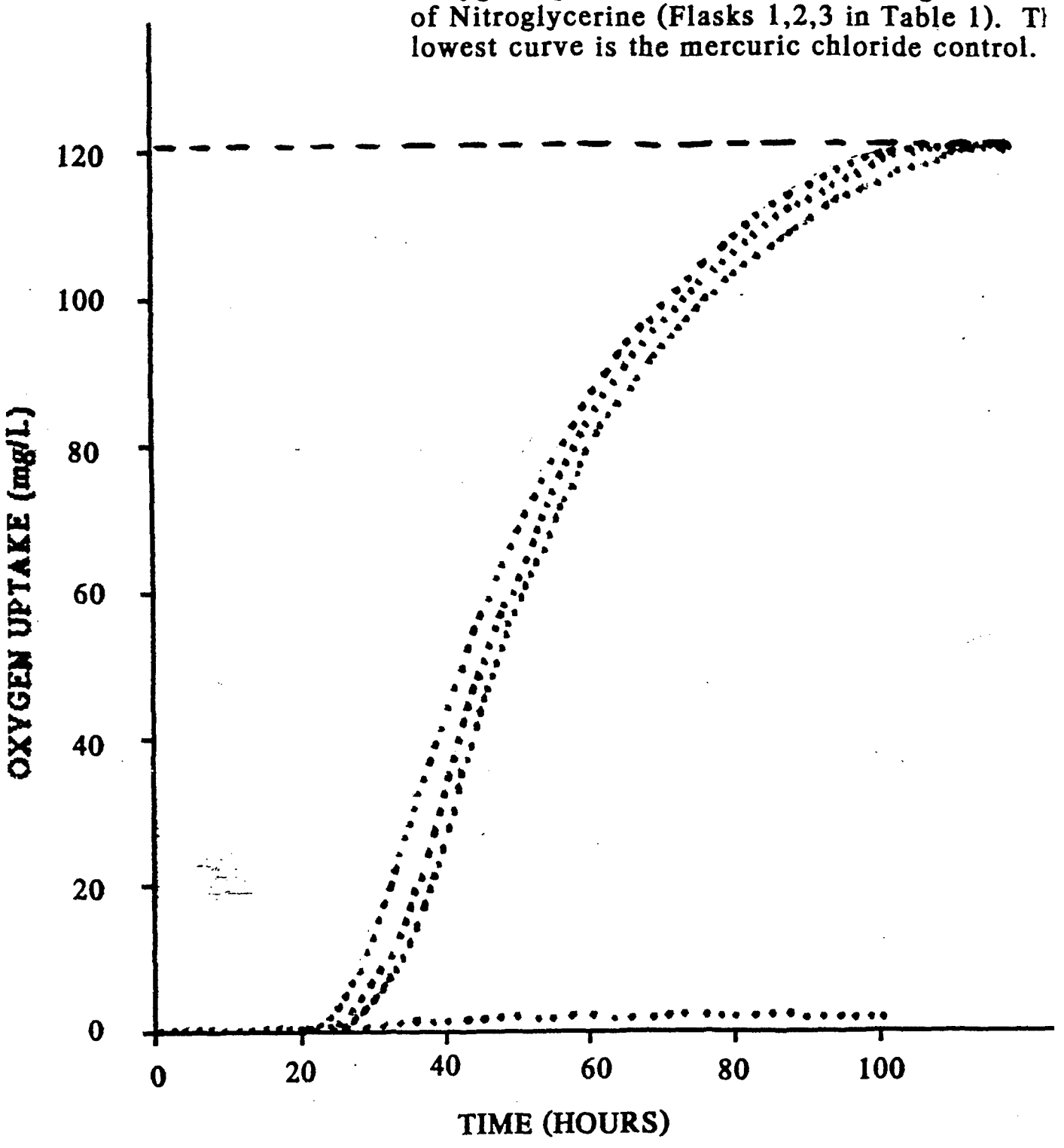


FIGURE 2: Oxygen Uptake curves for aerobic degradation of Nitroglycerine with Aniline (Flask 10 in Table 1). The lowest curve is the mercuric chloride control.

Note that aniline degradation was delayed, due to toxicity effects. Also the aniline did not degrade completely.

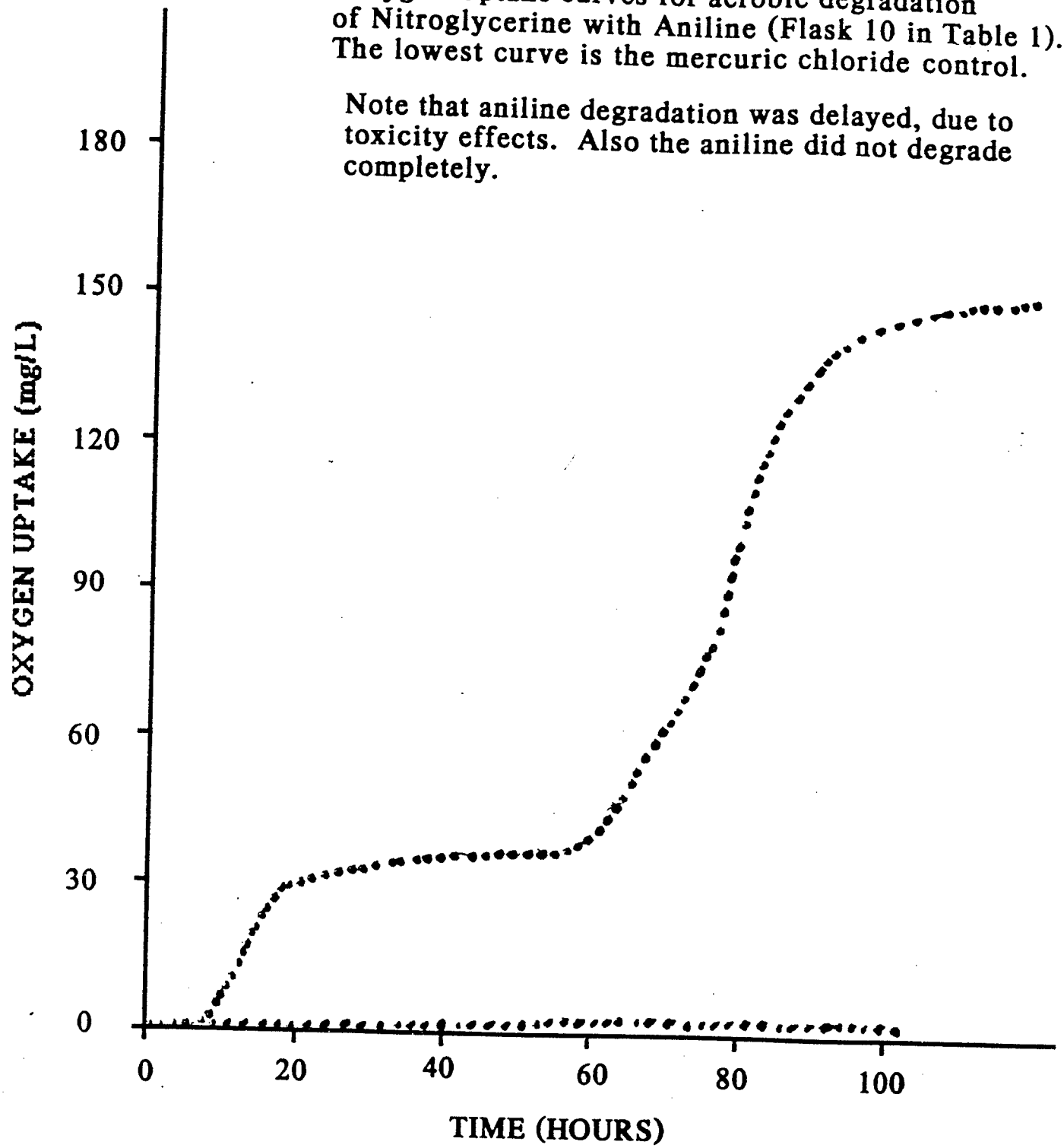


FIGURE 3: Oxygen uptake curve for PGDN solution. Note that oxygen uptake was very small for all concentrations of PGDN. The three curves represent the average of flasks (1,2,3) (4,5,6) and (7,8,9) [Refer Table 1]. No oxygen uptake was measured for flasks 10 &11. Hence, no aniline degradation occured due to toxicity of PGDN.

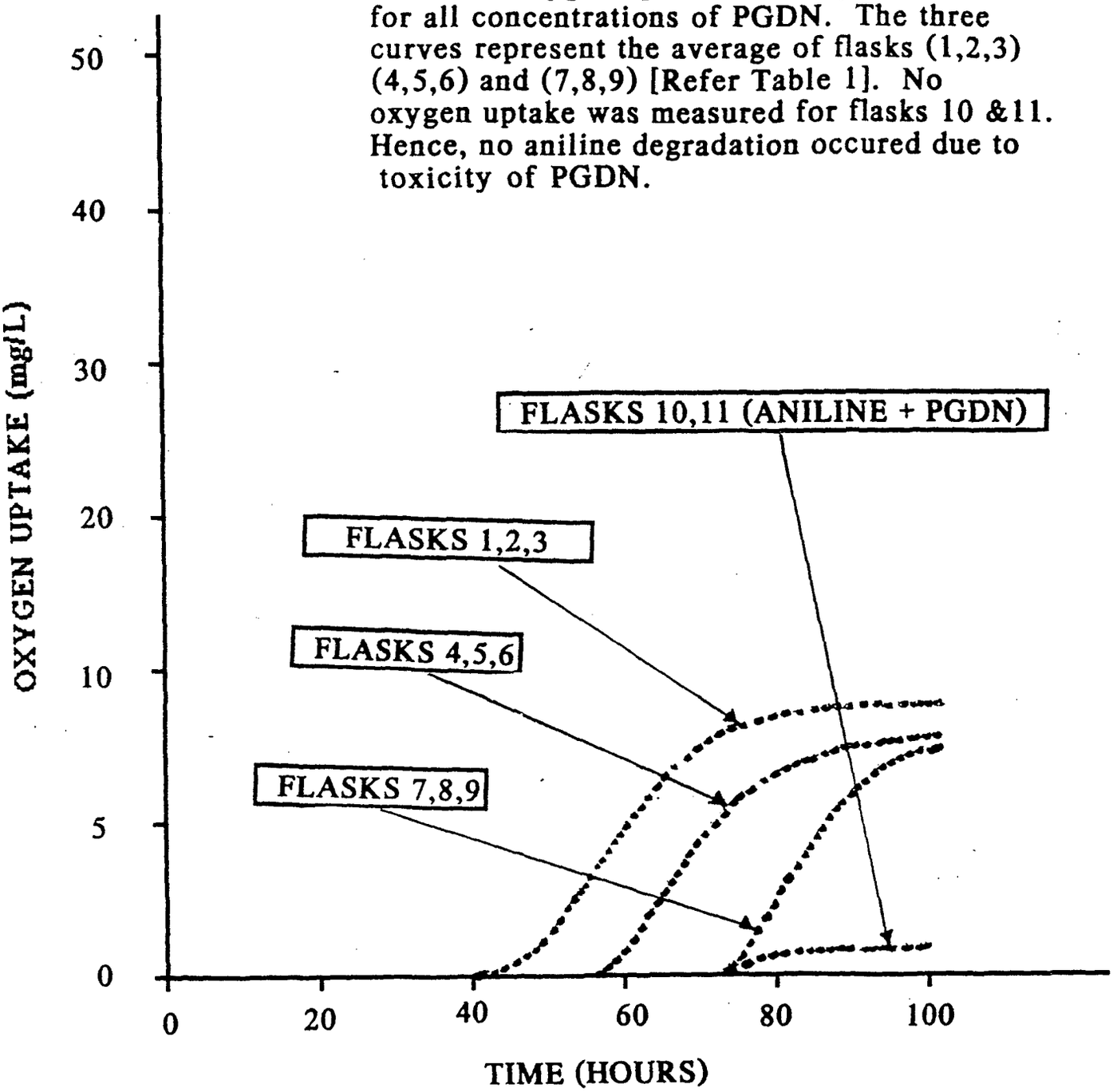


FIGURE 4: Oxygen uptake curve for TMETN solution. Note that oxygen uptake was very small for all concentrations of TMETN. The three curves represent the average of flasks (1,2,3) (4,5,6) and (7,8,9) [Refer Table 1]. No oxygen uptake was measured for flasks 10 &11. Hence, no aniline degradation occured due to toxicity of TMETN.

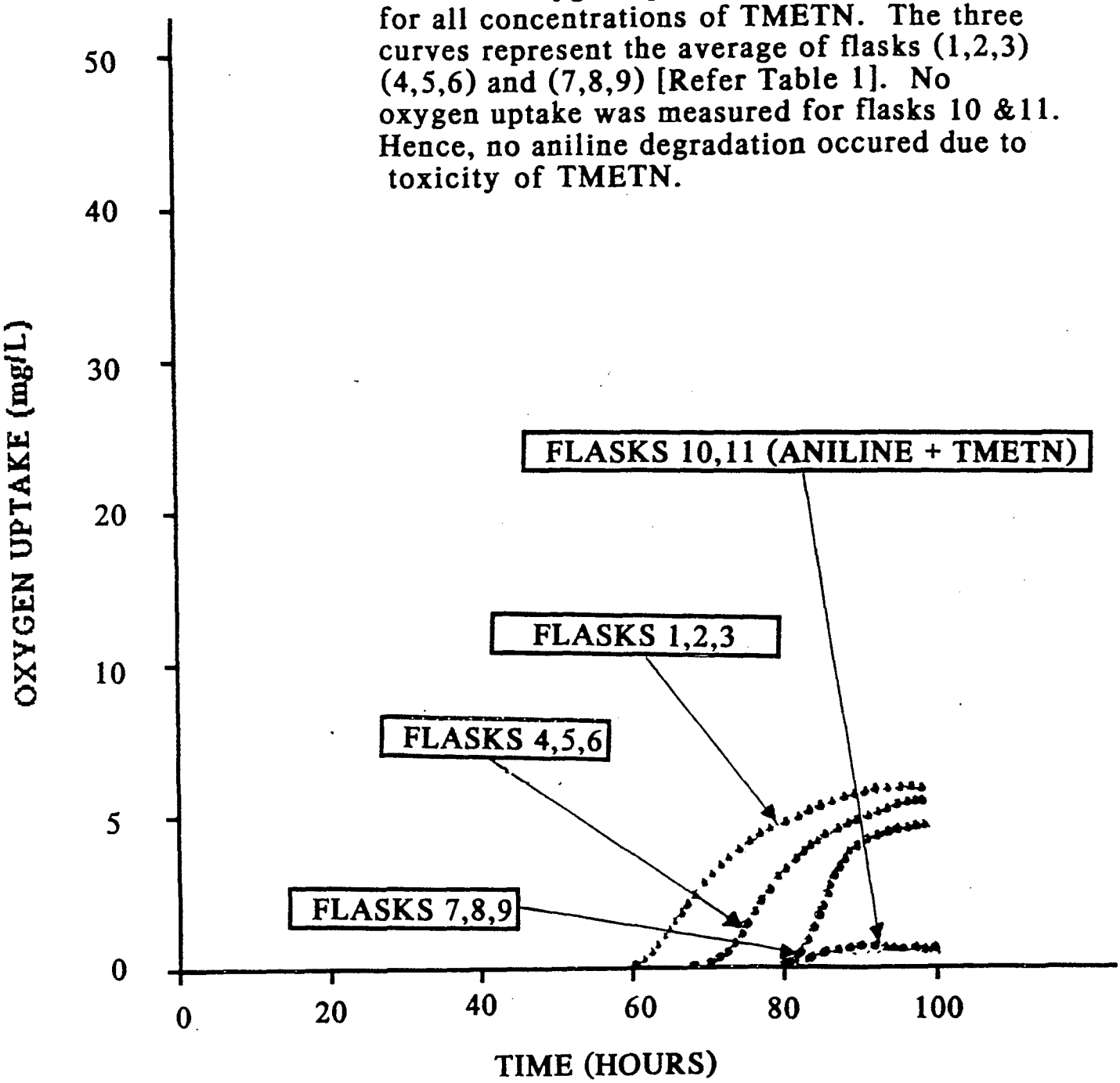


Figure 5. Oxygen uptake curves for aerobic degradation of nitroglycerine (NG) and mixture of NG with PGDN and TMETN.

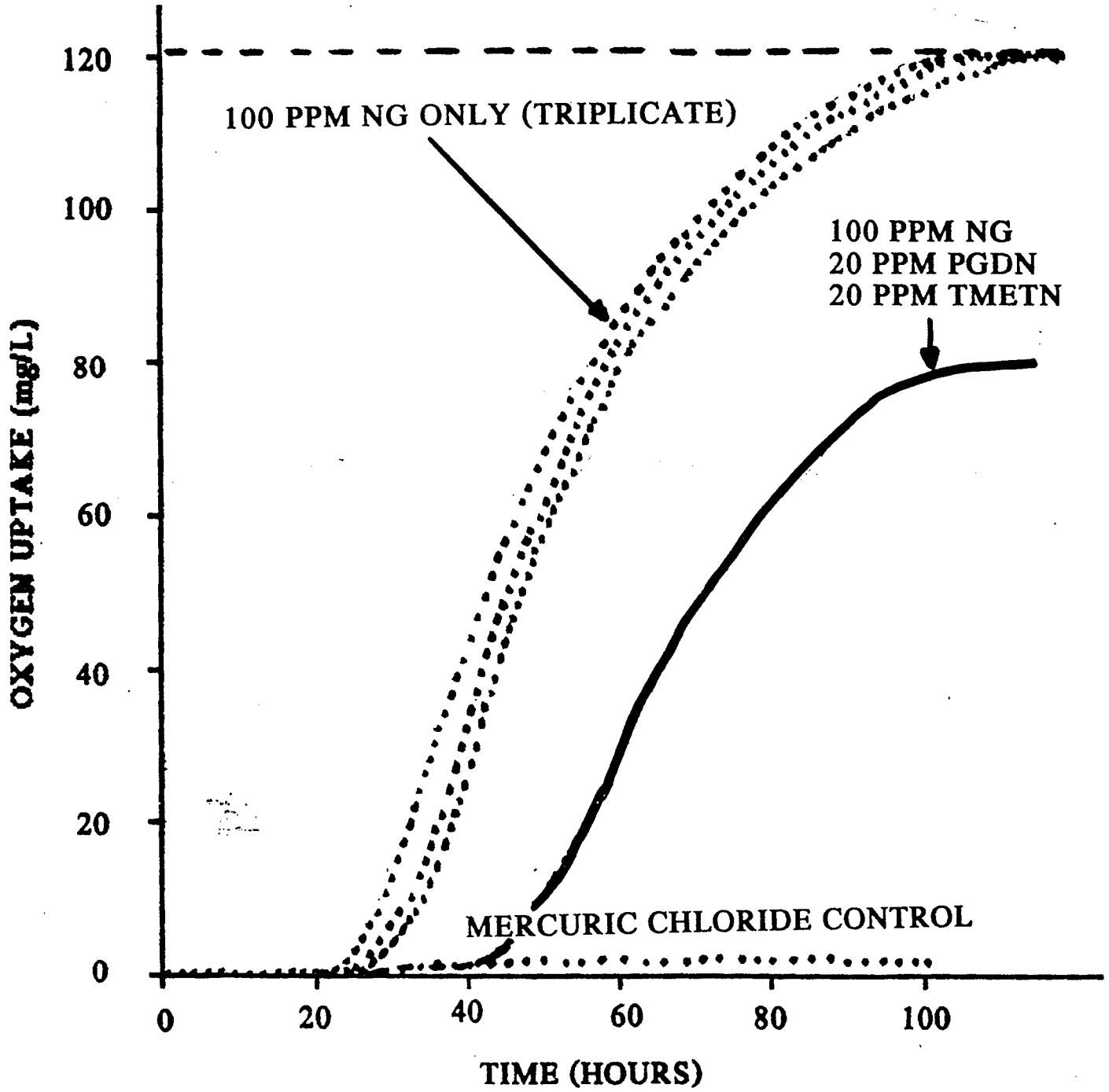


Figure 6. Schematic of the master culture reactor.

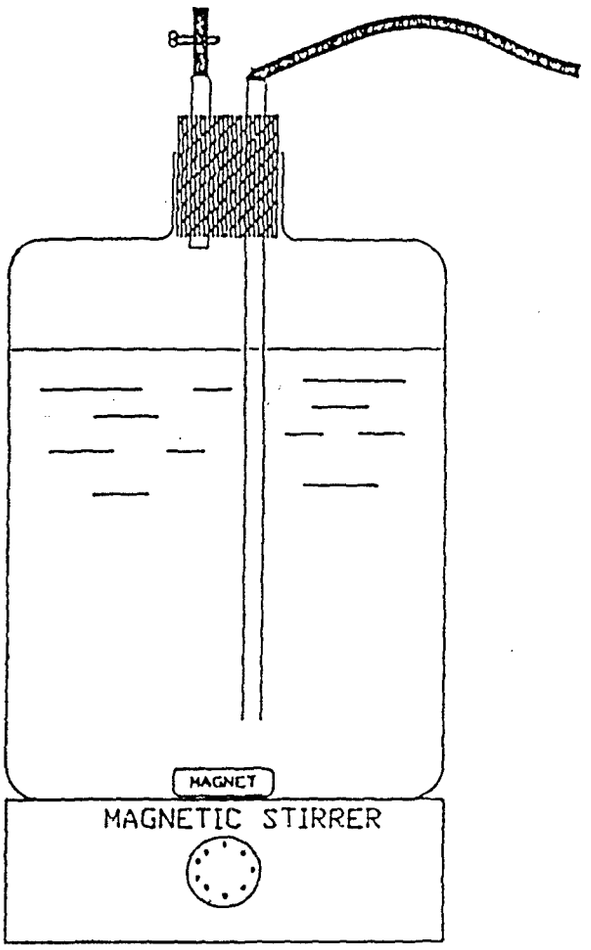


Figure 7. Standard calibration curve for analysis of acetic acid in the anaerobic master culture reactor.

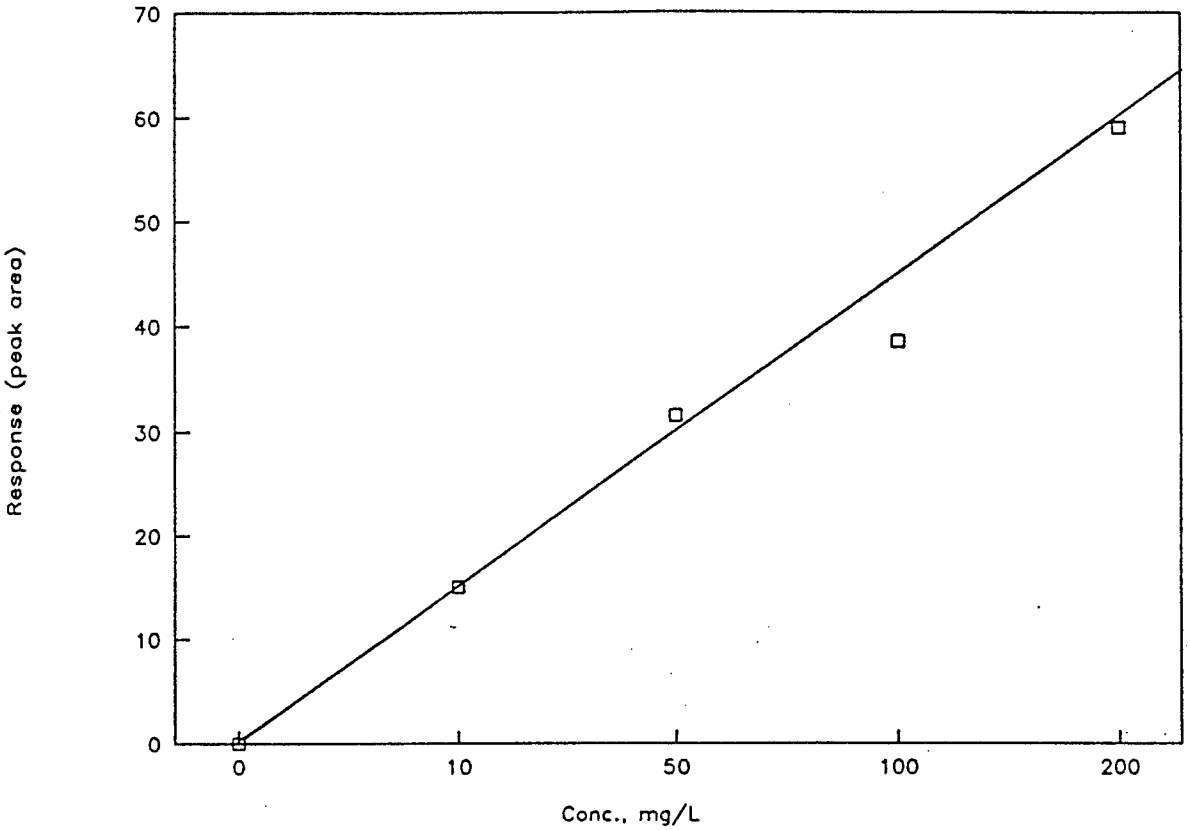


Figure 8. Standard calibration curve for analysis of propionic acid in the anaerobic master culture reactor.

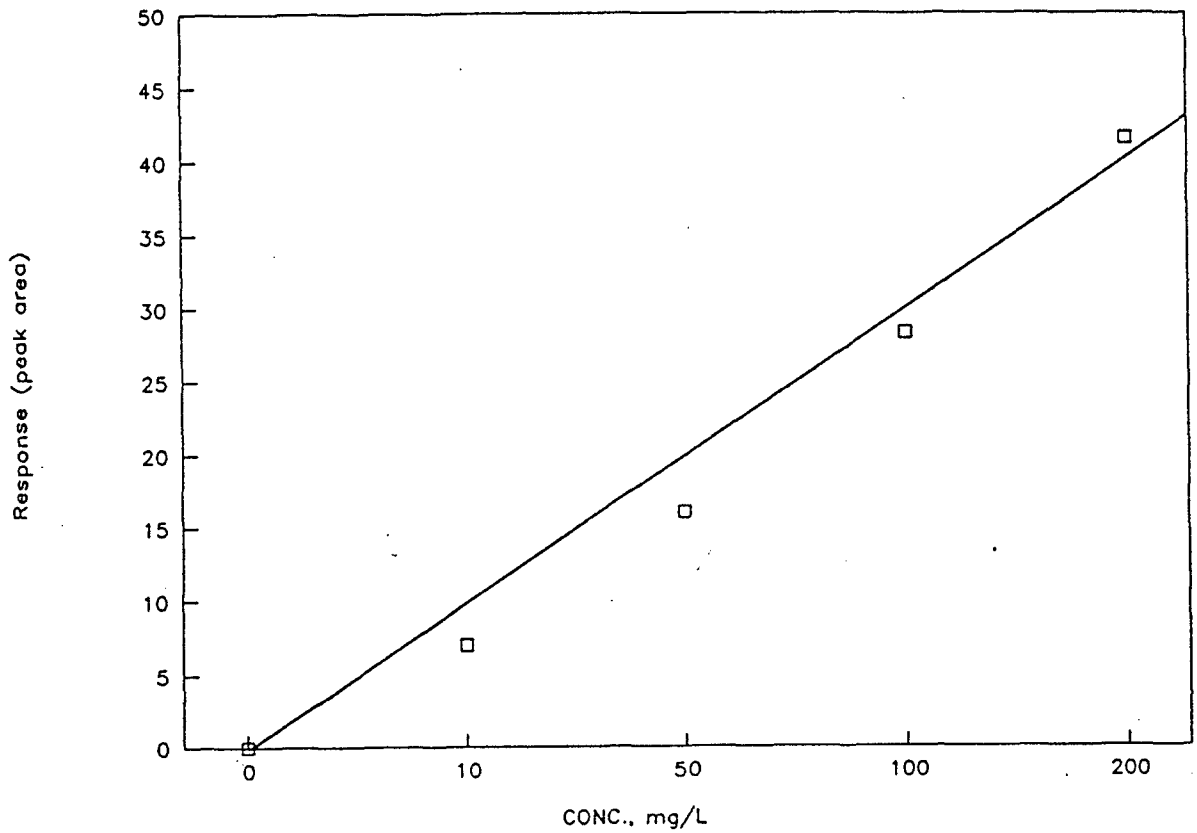


Figure 9. Standard curves obtained using HPLC analysis for nitrate ester compounds.

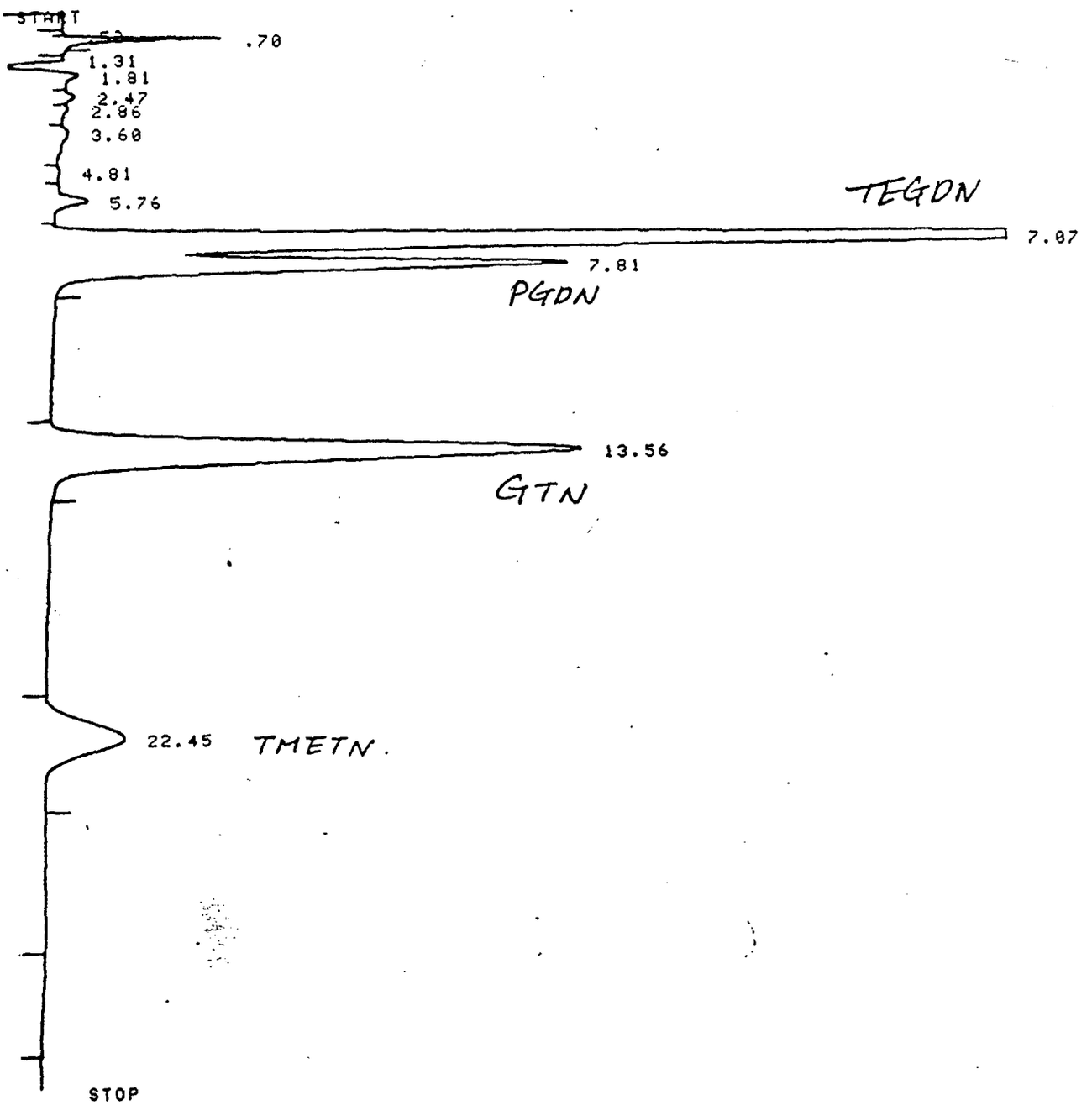


Figure 10. Anaerobic serum bottle results for nitroglycerine.

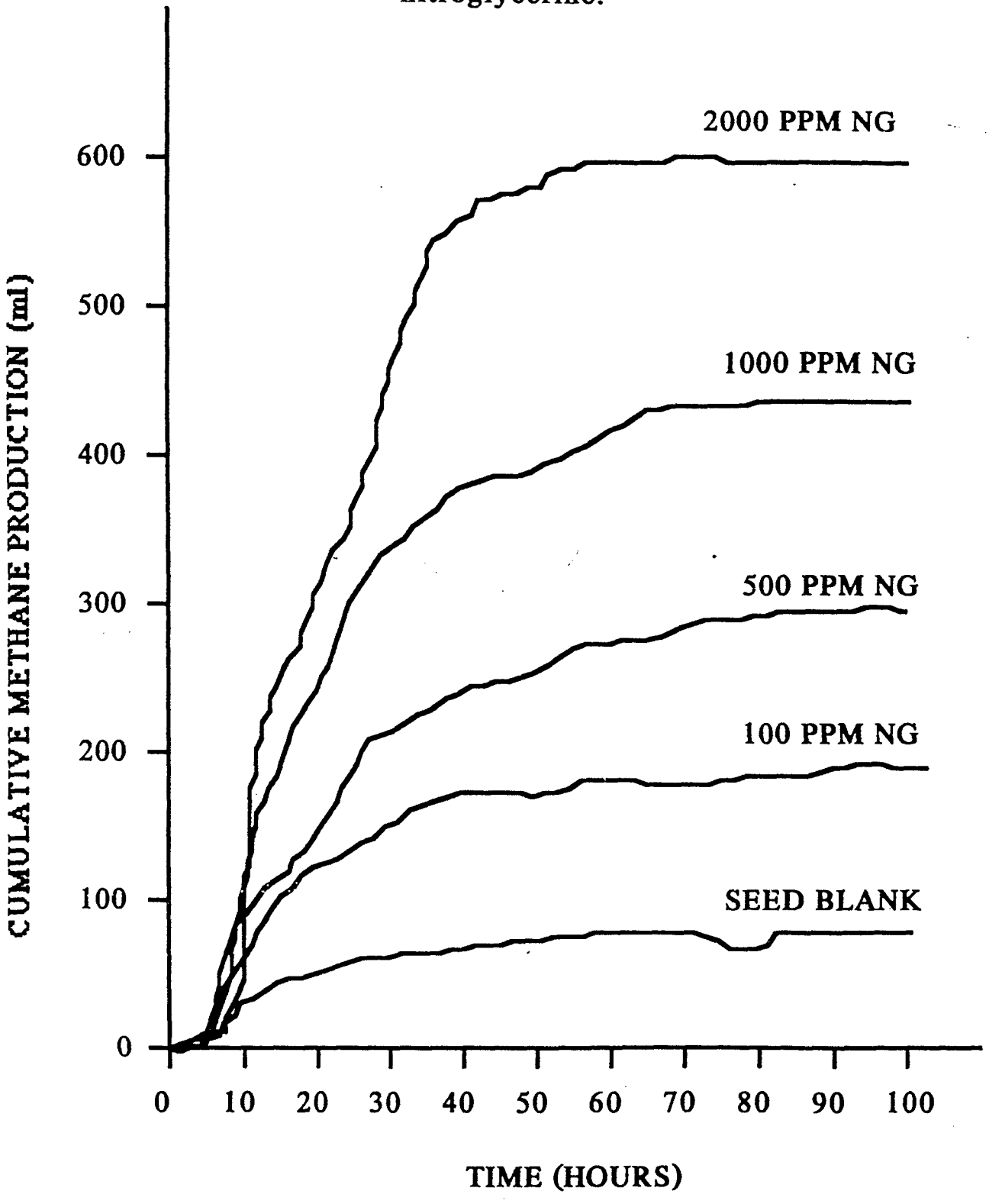


Figure 11. Anaerobic serum bottle results for PGDN.

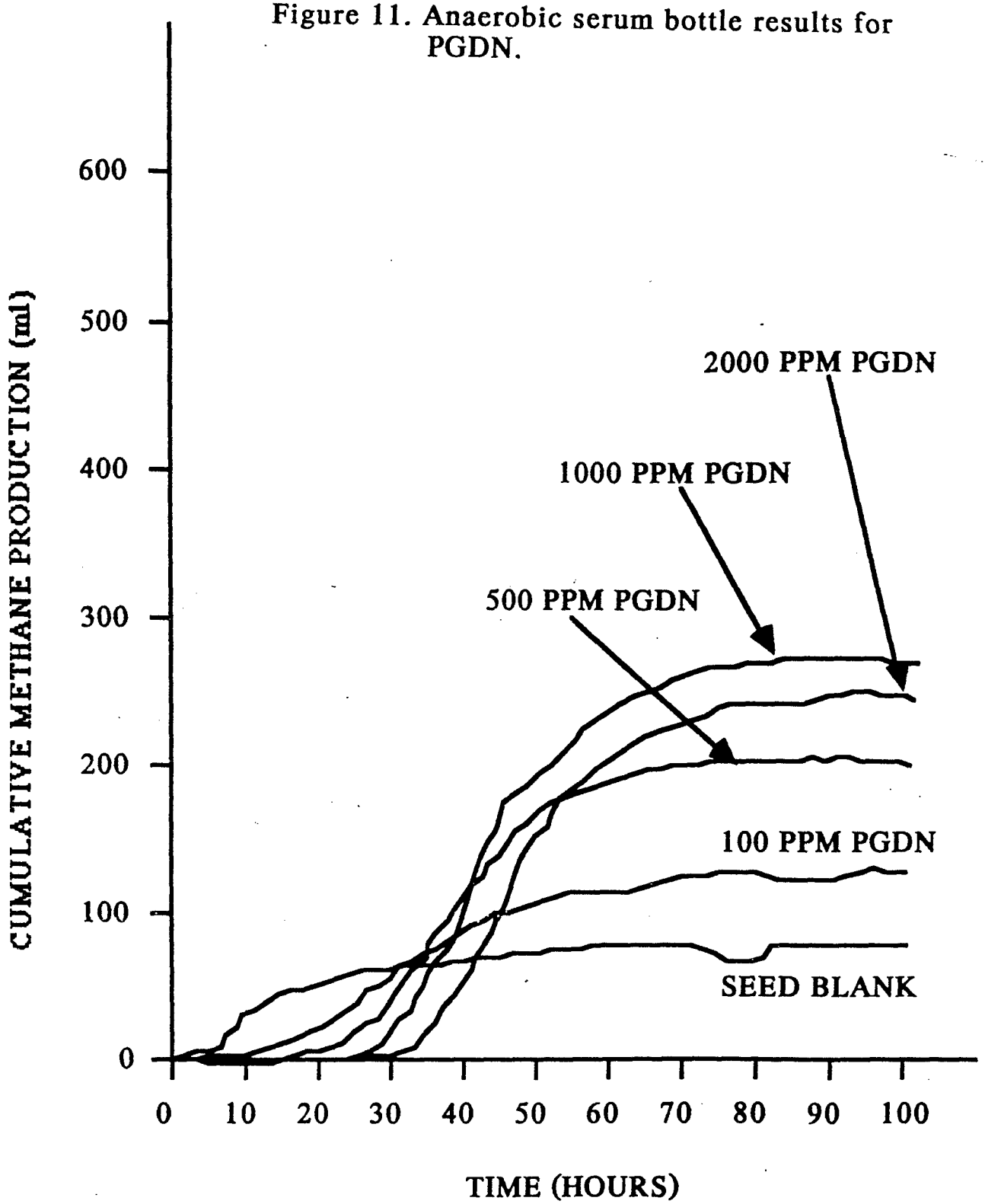


Figure 12. Anaerobic serum bottle results for TMETN.

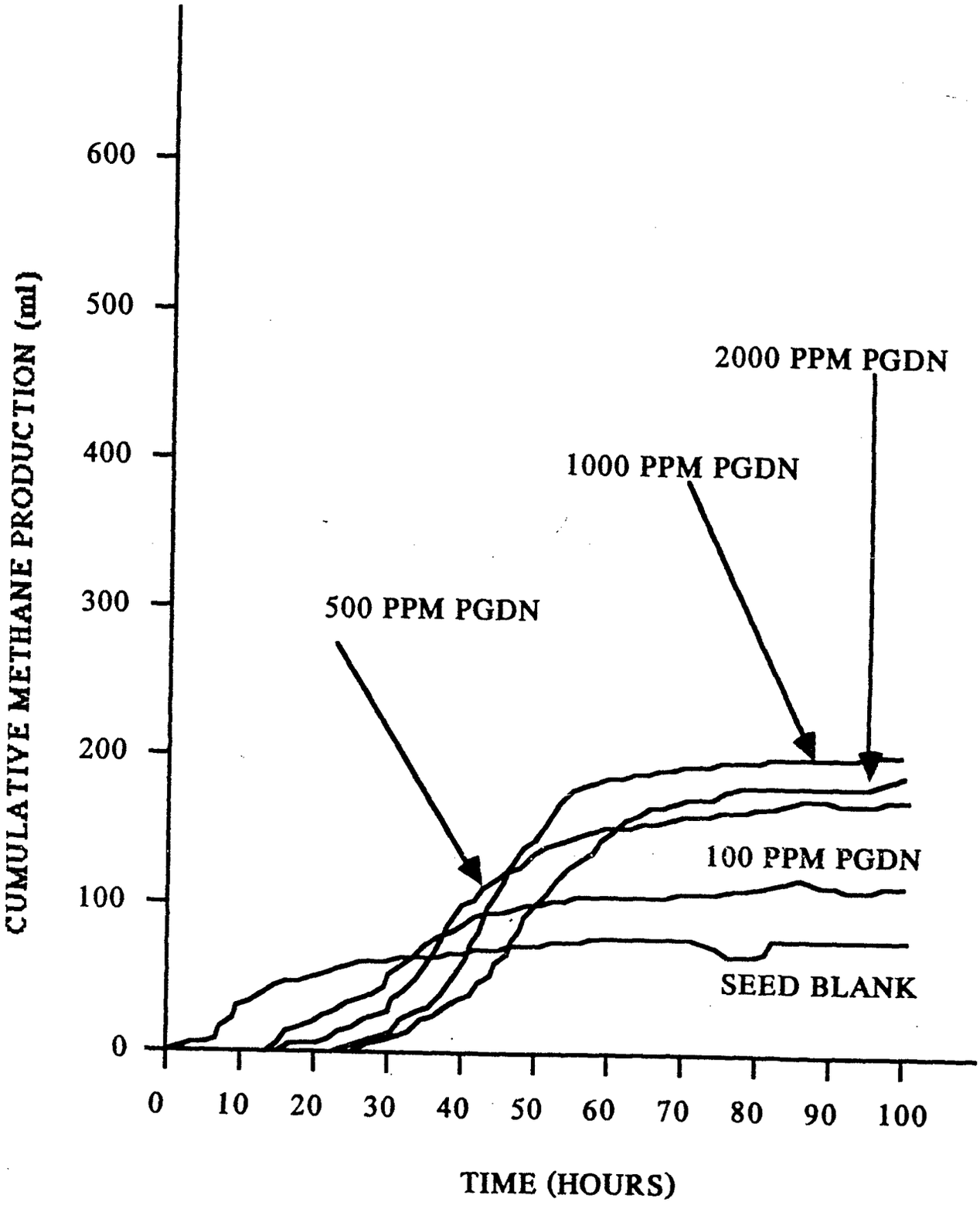


Figure 13. Gas production data for a mixture of NG, PGDN and TMETN.

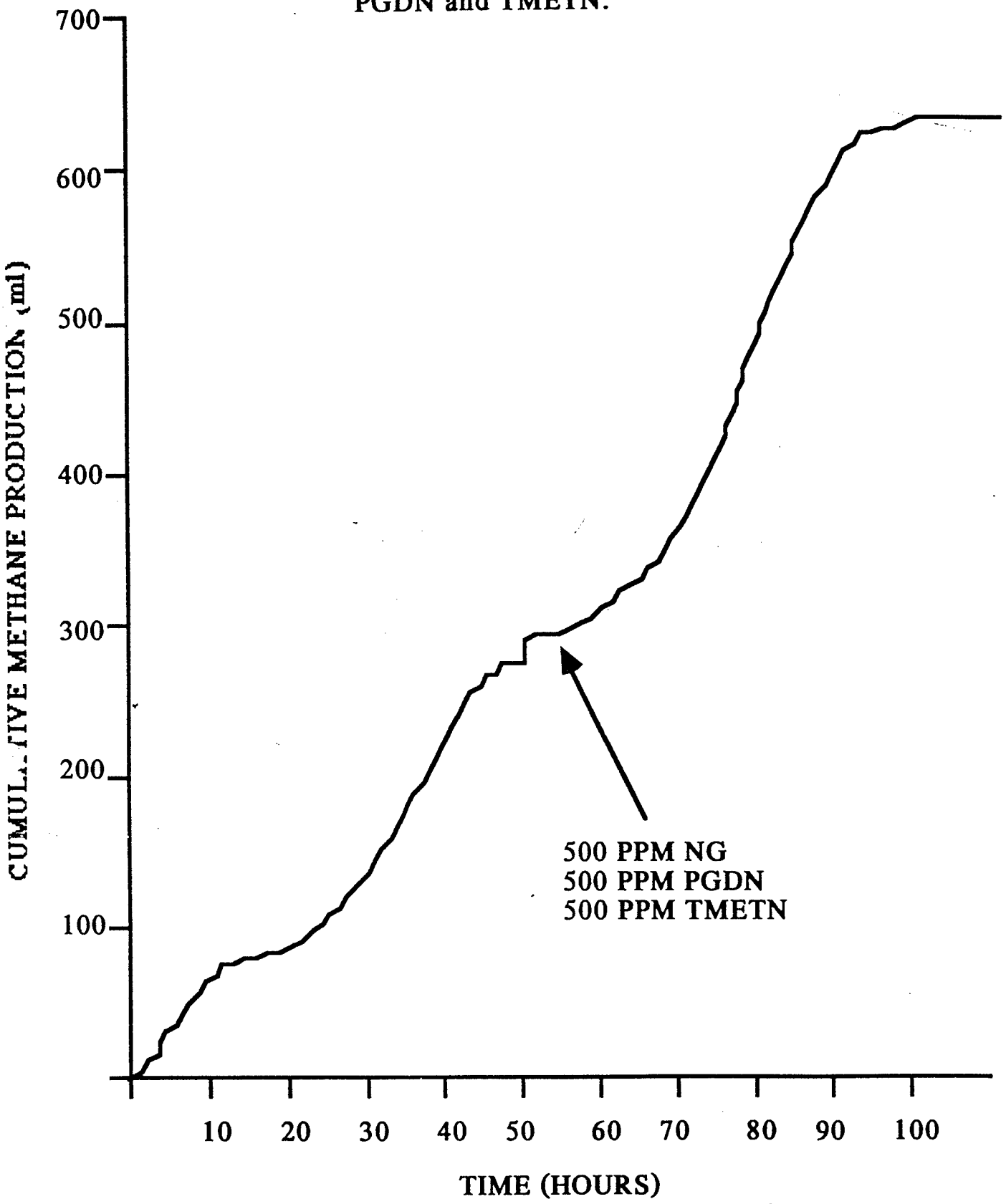


Figure 14. Cumulative methane production for TMETN water at a concentration of 1500 ppm.

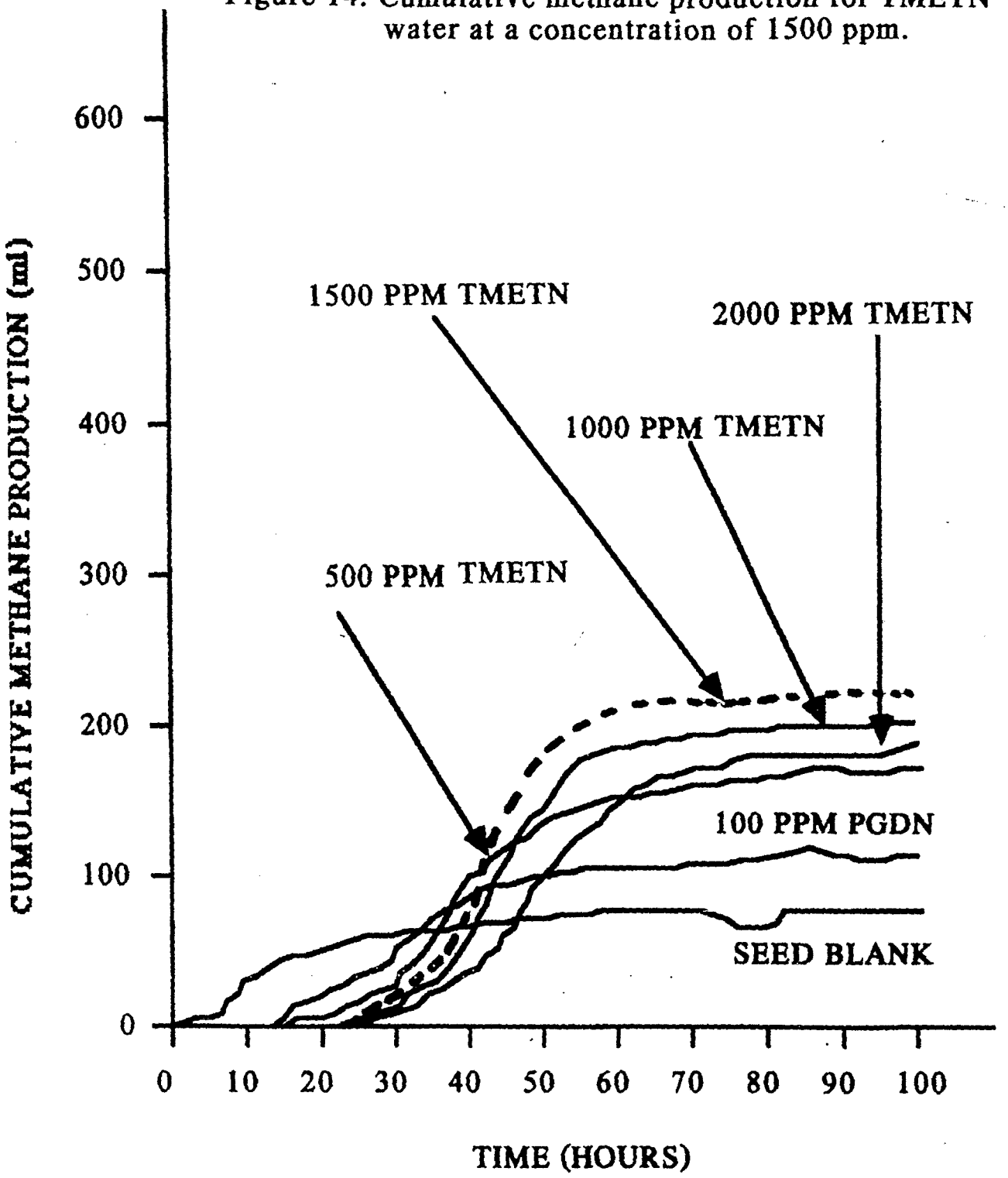


Figure 15. Changes in the concentration of NG with time in the anaerobic serum bottle.

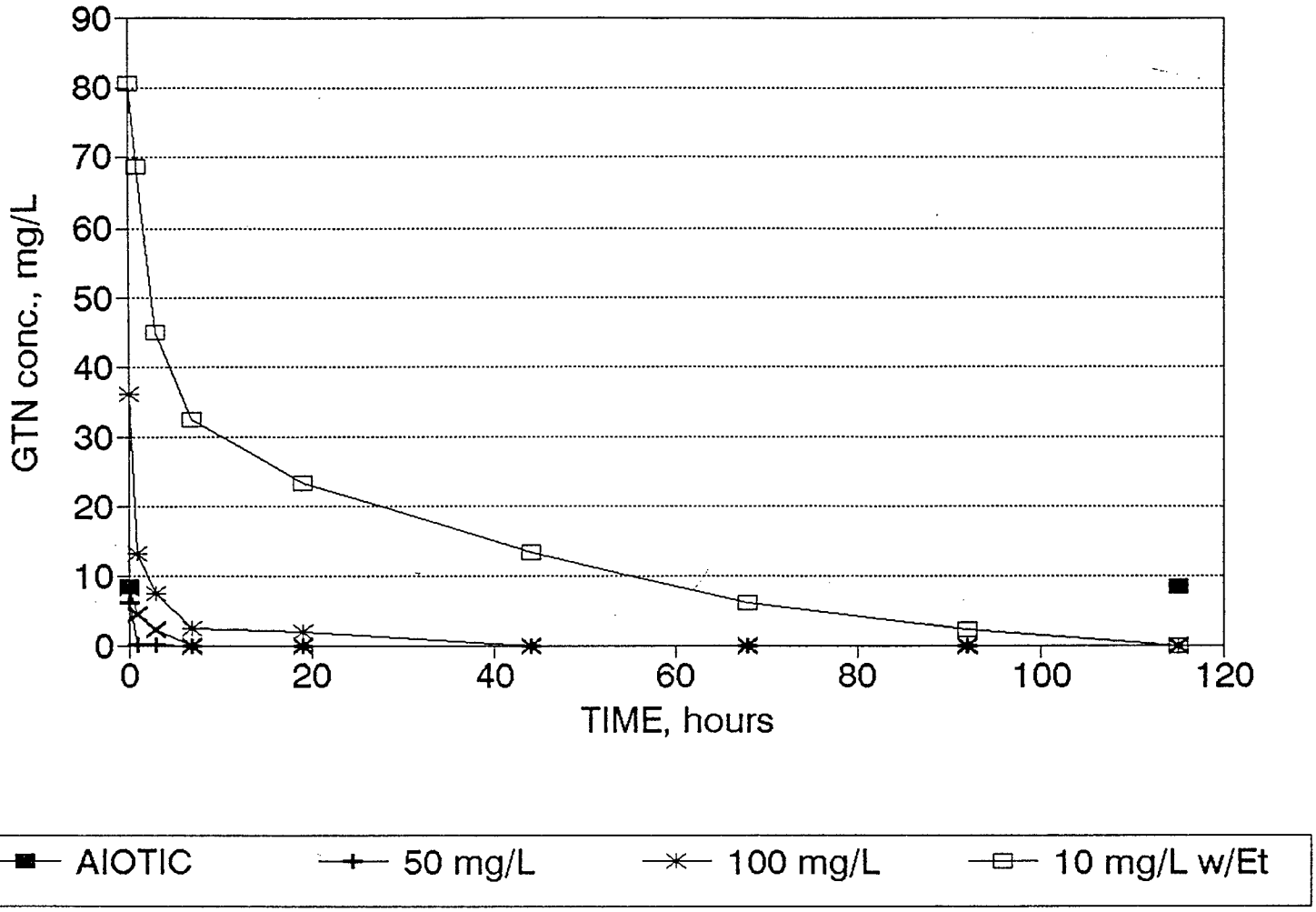


Figure 16. Response peak area versus time for NG at an initial concentration of 50 mg/L.

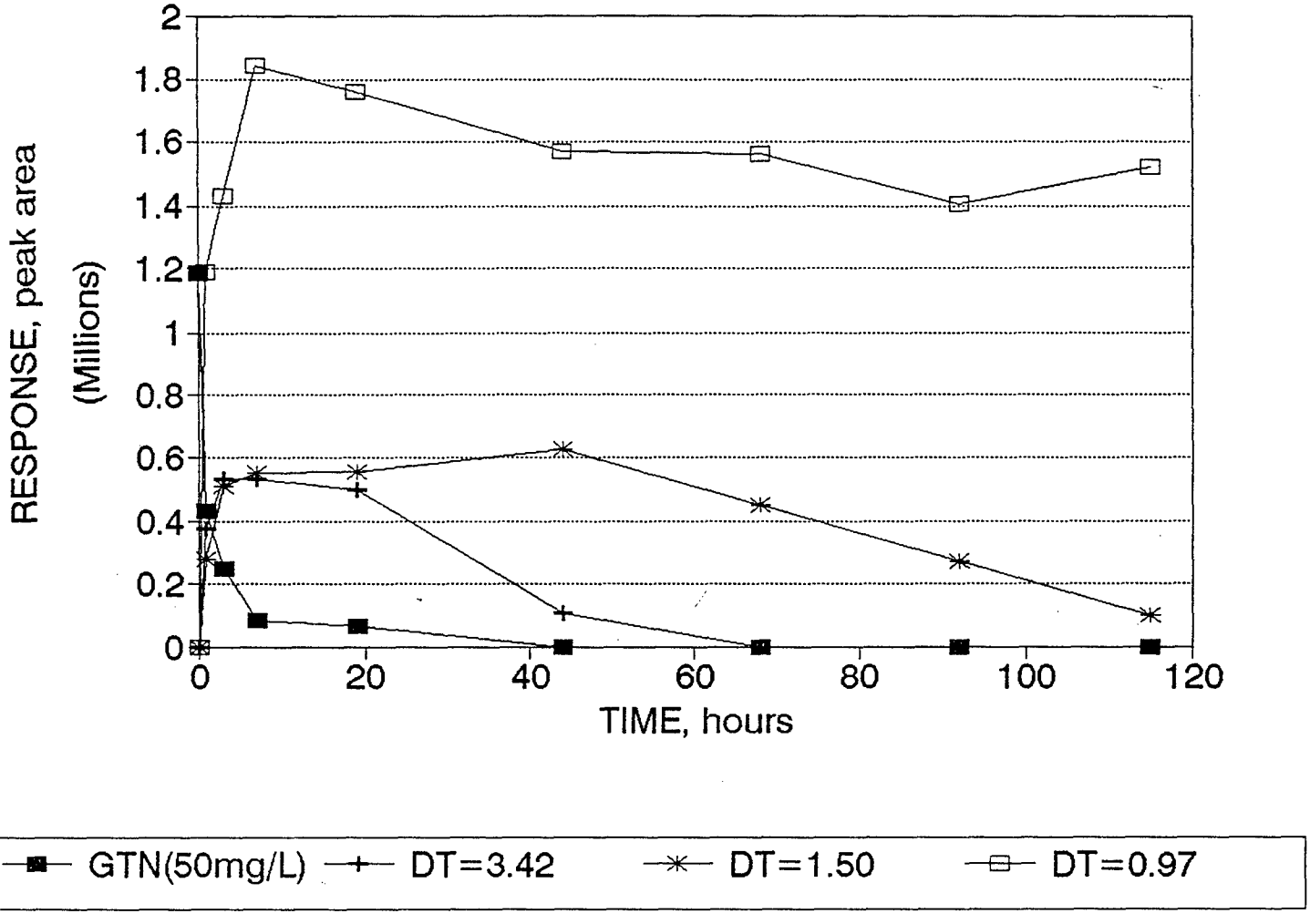


Figure 17. Response peak area versus time for NG at an initial concentration of 100 mg/L.

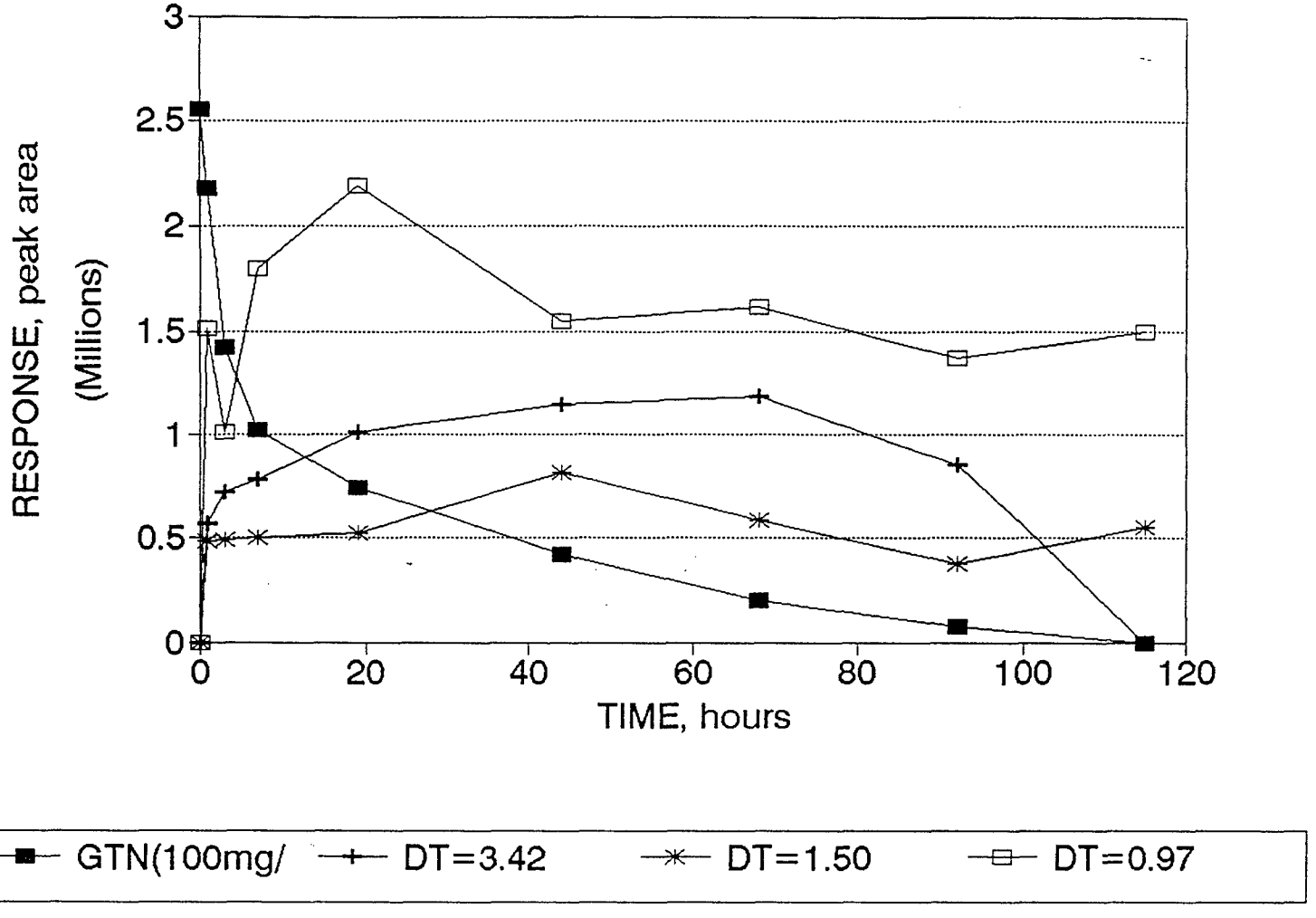
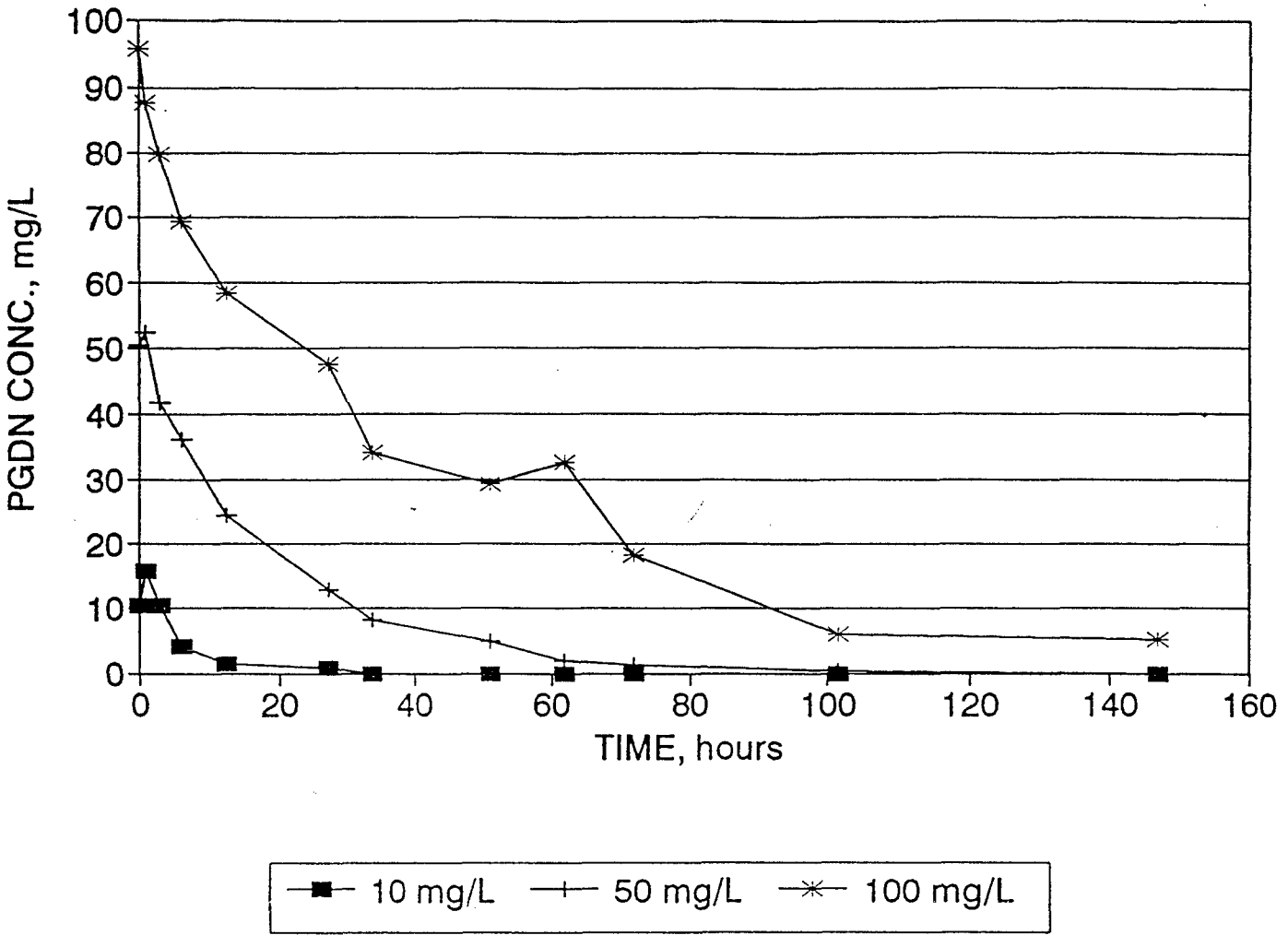


Figure 18. Kinetic data for PGDN solution.



APPENDIX 1. SAIC METHOD FOR HPLC ANALYSIS

NITROGLYCERINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1.0 SCOPE AND APPLICATION

1.1 Method 8332 provides two procedures; a qualitative procedure for the presence or absence of nitroglycerine, and a quantitative analysis for determining the concentration of Nitroglycerine. The Method is used to determine the concentration of the following compounds in waste waters, and may be applicable to other matrices:

Compound Name	CAS No.[a]
Nitroglycerine	

[a] Chemical Abstract Services Registry Number.

2.0 SUMMARY OF METHOD

2.1 Samples are streaked or spotted on a thin layer chromatography plate and developed in a solvent tank. The spots are developed using one of two spray on reagents, and a qualitative determination is made for the presence or absence of nitroglycerine.

2.2 Samples are injected onto a high performance liquid chromatograph column (HPLC) and the nitroglycerine concentration is quantitated using a UV detector.

3.0 INTERFERENCES

(If there are no specific interferences listed, put in our standard boilerplate language here.)

4.0 APPARATUS AND MATERIALS

4.1 HPLC system

4.1.1 HPLC (isocratic) - equipped with a pump, a direct injection port or 20 µL loop injector and a 214 nm UV detector.

4.1.2 Column - Waters Radial - Pak CN, 10 µm particle size (or equivalent).

4.1.3 Integrator.

SENT BY:SAIC Falls Church ; 8- 3-92 ; 1:40PM ; 7038214719→ 513 569 7787:# 2

4.2 Injection syringe.

4.3 Thin-layer chromatography (TLC) system

4.3.1 TLC plate, Analtech silica gel GHL (Catalog # 11511), or equivalent.

4.3.2 Device (syringe, capillary pipet or other) for spotting TLC plate.

4.3.3 TLC developing tank.

4.3.4 Sprayer.

5.0 REAGENTS

5.1 Reagent grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

5.2 Organic-free reagent water - All references to water in this method refer to organic-free reagent water, as defined in Chapter One.

5.3 Solvents

5.3.1 Acetonitrile, CH_3CN.

5.3.2 1,2-Dichloroethane, $Cl_2CH_2CH_2Cl$.

5.3.3 Carbon tetrachloride, CCl_4.

5.3.4 Ethanol (absolute), CH_3CH_2OH.

5.3.5 Acetone, CH_3COCH_3.

5.3.6 Tetrahydrofuran, C_4H_8O.

5.4 Diphenylamine, $(C_6H_5)_2NH$.

5.5 alpha-Naphthylamine, $C_{10}H_7NH_2$.

5.6 Sulfanilic acid, $4\text{-}(H_2N)C_6H_4SO_3H$.

5.5 Sulfuric acid, H_2SO_4.

5.5 Potassium Hydroxide, KOH.

5.9 Acetic acid (30% w/w?), CH_3CO_2H.

5.10 Nitroglycerine - "Nitrostat" tablets (nominal concentration =

0.4 mg/tablet), available from Warner Lambert/Parke Davis. Certificate of Analysis for any current lot is available from Warner Lambert, Lititz, Pa. (717) 626-2011.

5.11 HPLC mobile phase eluant - 60% Acetonitrile/40% Organic-free reagent water.

5.12 HPLC calibration standards - Dissolve five nitroglycerine tablets in HPLC mobile phase, using a 500 mL volumetric flask. Dilute to volume with mobile phase. The resulting standard will contain 4 mg/L nitroglycerine. Similarly, prepare 8 mg/L and 12 mg/L nitroglycerine standard solutions. Use the concentration listed on the "Nitrostat" Certificate of Analysis to calculate the concentration of the standards to the second decimal place.

5.13 TLC developing solvent - 20% dichloroethane/80% carbon tetrachloride.

5.14 TLC overspray - Prepare a solution of 5% diphenylamine in ethanol. For very weak spots, a solution of 5% diphenylamine in concentrated sulfuric acid may be required.

5.15 Alternative TLC overspray (Greiss reagent) -

1. Alcoholic KOH (2% w/w)
2. 1% (w/w) sulfanilic acid in acetic acid (30%)
3. 1% (w/w) alpha-naphthylamine in acetic acid (30%)

5.16 TLC standard - Use double-base smokeless powder (e.g. Hercules Red Dot, Bullseye, or Winchester-Western ball powder) available at any gun shop. Dissolve in acetone, acetonitrile, or THF.

6.0 SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1 See the introductory material to this Chapter, Organic Analytes, Section 4.1.

7.0 PROCEDURE

7.1 Chromatographic Conditions (Recommended):

Flow rate: 1.0 mL/min.
UV Detector: 214 nm.
Injection: 20 µL (for 4 to 12 mg/L nitroglycerine).

Chromatographic conditions should be established to give a retention time for nitroglycerine of approximately 5.1 min.

7.2 Initial Calibration - Analyze three 20 µL injections for each nitroglycerine standard solution (4, 8, and 12 mg/L). Calculate a correlation coefficient, slope, and zero intercept from the regression analysis of the data points (peak area vs. concentration). (Why not go with our usual 5-point calibration curve per Method 8000? Do we really need triplicate injections?)

7.3 Make three 20 µL injections for each sample or sample dilution. If

the response for the sample exceeds that of the initial calibration range, the sample must be diluted. Calculate sample concentrations using the slope and the intercept figures from the regression analysis of the standards. (Triplicate injections are unnecessary.)

7.4 Thin-layer chromatography procedure:

7.4.1 Add a quantity of the developing solvent mixture to the developing tank that will be sufficient to continuously wet the edge of the TLC plate. Cover the tank tightly, and allow the vapor phase to equilibrate (several hours).

7.4.2 Streak or spot the sample near the edge of the plate, but above the area that will be immersed in the developing solvent. Allow the streaked area to dry completely.

7.4.3 Streak or spot the standard solution next to the sample spot, at the same distance from the edge of the plate as the sample spot. Allow the streaked area to dry completely.

7.4.3 Stand the plate in the equilibrated developing tank and cover tightly. Let the plate develop until capillary action has carried the solvent nearly to the top of the plate.

Note: It may be necessary to adjust the developing time to achieve suitable separation of the nitroglycerine from interfering sample components.

7.4.4 Remove the plate from the tank and air dry.

7.4.5 Develop the plate using the procedure in Section 7.4.5.1 or 7.4.5.2.

7.4.5.1 Spray the developed plates with a 5% solution of diphenylamine in ethanol. After spraying the developed TLC plates, expose the plates to UV light (longwave, shortwave, or both simultaneously) for 15-30 minutes, until spots appear. For very weak spots, spray again with 5% diphenylamine in concentrated sulfuric acid.

7.4.5.2 Spray the developed plates with Greiss reagent (Section 5.15) and warm the plates in a 100°C oven for 5-10 minutes.

7.4.6 The presence of a spot at the same distance from the origin as the standard (Rf of approximately 0.4) indicates that nitroglycerine may be present. The absence of a spot in this location indicates that nitroglycerine is not present above the detection limit of the test.

8.0 QUALITY CONTROL

8.1 Refer to Chapter One and Method 8000 for specific quality control procedures.

9.0 METHOD PERFORMANCE

9.1 Method 8332 was tested by one laboratory using two industrial wastewater samples. The wastewaters were analyzed at two dilutions with three injections of each dilution. The results of these analyses are shown in Table 1.

10.0 REFERENCES

1. U.S. Department of the Treasury; Bureau of Alcohol, Tobacco and Firearms; Lab Number 88-N-0648 B.

TABLE 1.
SINGLE LABORATORY PRECISION

Nitroglycerine by Weight	
As parts per Million	As Percent
1036 ± 38	0.1036 ± 0.0038
952 ± 53	0.0952 ± 0.0053

The numbers shown are the average concentration plus or minus one standard deviation calculated for six analyses on each sample.

SECOND QUARTERLY REPORT

on

STUDIES ON THE BIODEGRADATION OF ORDANCE-RELATED HAZARDOUS WASTE

CATEGORY: MANUFACTURING OF ORDANCE AND ORDANCE COMPONENTS AND CHEMICALS

SUBMITTED TO: JOHN STACEY
Code 2810C
Naval Ordnance Station
Indian Head, MD 20640-5000

SUBMITTED BY: Dr. Rakesh Govind
Department of Chemical Engineering
Mail Location 171
University of Cincinnati
Cincinati, OH 45221

May 3, 1992

INTRODUCTION

The overall goal of the proposed study is to address the following areas for the following chemicals: Nitroglycerine, Glycerol trinitrate, Propylene glycol dinitrate, Triethylene glycol dinitrate and Trimethyl ethane trinitrate:

1. Determining factors to optimize biodegradation under laboratory and field conditions;
2. Use of immobilized microbes and enzymes for biodegradation and development of field bioreactors; and
3. Developing new technologies for treating wastewaters, groundwater, air and soil contamination.

PROJECT OBJECTIVES ADDRESSED IN THIS REPORT

The specific objective of this interim report is to determine anaerobic biodegradation kinetics for the following mixtures of compounds: Nitroglycerine, Propylene Glycol dinitrate (PGDN), and Trimethyloethane trinitrate (TMETN) using biological methane production. In the previous report biological methane production data was obtained for each individual compound and it was shown that anaerobically these compounds could be decomposed upto a concentration of 2000 ppm.

A mixture of all three compounds with a concentration of 500 ppm for each compound was synthesized and tested for anaerobic degradation. In addition, TMETN experiments were conducted at 1500 ppm in an attempt to determine the maximum concentration that can be degraded anaerobically before toxicity effects occur to the seed

culture.

Aerobic respirometric studies were conducted with radiolabelled NG in the presence of the other non-labelled PGDN and TMETN. The basic objective was to investigate if the presence of the other compounds would inhibit the degradation of NG.

EXPERIMENTAL STUDIES

Determination of Anaerobic Kinetics:

A standard batch anaerobic bioassay technique (Biological Methane Potential (BMP) and Anaerobic Toxicity Assay (ATA)) was used to determine the methane potential and the anaerobic toxicity of a mixture of Nitroglycerine, TMETN, and PGDN.

The BMP assay was conducted with Corning no. 1460, 250 ml reagent bottle and ATA assay was conducted with 125 ml reagent bottles with rubber septum caps of appropriate size. The bottles were gassed at a flow rate of approximately 0.5 l/min. with 30% CO2 and 70% N2, then stoppered and equilibrated at incubation temperature of 35 C prior to introducin samples, defined media and inoculum. The defined media contained nutrients and vitamins for mixed anaerobic cultures. Resazurin was added to detect oxygen contamination and sodium sulfate was added to provide a reducing environment. The final assay concentrations of nitrogen, phosphorus, and alkalinity were respectively: 12 mg/l as N, 19 mg/l as P and 2500 mg/l as CaCO3.

For BMP assay, inoculation was accomplished anaerobically by inserting a gas flushing needle into the neck of the media flask while adding 200 ml of seed organisms to 1800 ml of defined media.

A 20% by volume inoculum was used.

Samples were added to the serum bottles anaerobically, and experiments at a mixture concentration of 500 ppm for each of the three compounds were run in triplicate. A seed blank with no addition of chemical was also run in triplicate. In addition, experiments were repeated for TMETN to determine the upper limiting concentration for toxicity effects.

For ATA assay, a "spike" containing acetate and propionate in addition to the seed blanks was also added to each bottle. A control with only the spike was also prepared. Each bottle contained 2 ml of acetate-propionate solution containing 75 mg acetate and 26.5 mg popionate.

Gas volume production was measured with the help of a calibrated pressure transducer. The composition of the gas was also measured using a Gas Chromatograph.

Preparation of Chemical Stock Solution and analytical methods:

The water sample, sent by the Navy, was extracted with ethyl ether in a continuous extractor for 24 hours, which very efficiently extracted essentially all the nitrate ester compounds. The extracts were concentrated by evaporation of solvent, at room temperature. Quantitative analysis of the nitrate ester compound was made by using a Hewlett Packard GC/MS and a Varian 4100 HPLC in conjunction with a silica column (Varian Micropak Si, 10 um) 50 cm in length with 2.2 mm ID. Analysis was conducted at 220 nm with a back pressure of 1,800 lb/sq. in.

The analyses were calibrated using the standards, sent by the Navy.

Use of Electrolytic Respirometry for Aerobic Kinetics:

Electrolytic Respirometry studies were conducted using an automated continuous oxygen measuring Voith Sapromat B-12 (Voith-Morden, Milwaukee, WI). The system consists of a temperature controlled bath which contains the measurin units, a recorder for digital indication, a plotter for direct presentation of the oxygen uptake curves of substrates, and a cooling unit for conditioning and continuous recirculation of water bath contents. The system used has 12 measuring units each connected to a recorder. Each unit, consists of a reaction vessel, with a carbon dioxide absorber (2N pottasium hydroxide solution) mounted in a stopper, an oxygen generator, and a pressure indicator. The unit is sealed from the atmosphere, and hance fluctuations in baromatric pressure does not affect the results. The reactor contents are well stirred by a manetic stir bar, thus ensuring effective gas exchange. Biodegradation of the organic contents in the reactor vessel, creates carbon dioxide, which is absorbed by the potassium hydroxide solution, thereby creating a negative pressure in the flask. This decrease in reactor vessel pressure trigers the pressure sensor which switches the oxygen generator. Oxygen is generated by electrolysis of a copper sulfate -sulfuric acid solution. The oxygen flows into the reactor vessel, thereby restoring the oxygen content in the headspace. The amount of oxygen generated is monitored by measurement of the voltage

supplied to the electrolytic unit, and the cumulative oxygen generated per unit volume of the reactor flask contents, is plotted as the oxygen uptake curve.

The nutrient solution is made as per Organization of Economic Cooperation and Development (OECD) guidelines. It contains 10 ml of solution A and 1 ml of each of the solutions B to F per liter of synthetic medium : solution A - KH_2PO_4 8.5 g, K_2HPO_4 21.75 g, $Na_2HPO_4.2H_2O$ 33.4 g, and NH_4Cl 2.5 g; solution B - $MgSO_4.7H_2O$ 22.5 g; solution C - $CaCl_2$ 27.5 g; solution D - $FeCl_3.6H_2O$ 0.25 g; solution E - $MnSO_4.4H_2O$ 39.9 mg, H_3BO_3 57.2 mg, $ZnSO_4.7H_2O$ 42.8 mg, $(NH_4)_6Mo_7O_{24}$ 34.7 mg and $FeCl_3$.EDTA 100 mg; and solution F - Yeast extract 150 mg. The chemicals for each of the solutions A, B, C, D, E and F, are dissolved in 1000 ml of deionized water. The solution D is freshly prepared immediately before the start of an experiment.

The microbial inoculum was obtained from the acclimated culture reactor by centrifuging the mixed liquor. The dry weight of biomass was measured by drying samples, in duplicates, of 1 ml, 2 ml, and 3 ml at 105 C overnight. A concentration of 30 mg/l of sludge as dry weight was used in each reactor vessel. The total volume of the synthetic medium in the 500 ml capacity reactor vessels was brought up to a final volume of 250 ml.

Aerobic studies were conducted with NG concentration of 100 ppm and PGDN and TMETN concentration of 20 ppm each. 3000 dpm of radiolabelled NG was used in the mixture. The reason for using 20 ppm each of PGDN and TMETN was due to the fact that earlier studies had found that these compounds were toxic to the aerobic culture.

Samples were collected from the flasks and analyzed periodically. The oxygen consumption data was used as a guide to ensure that an adequate number of samples had been collected and that they have been spaced in time correctly.

Samples were acidified and mixed to separate the $^{14}CO_2$. One portion of the sample was analyzed directly for radioactivity and another was centrifuged before analysis. The latter sample indicated the amount of unreacted substrate whereas the difference between the two gave the amount of cell synthesis. The $^{14}CO_2$ liberated from the samples was trapped in a double-vial and analyzed by scintillation counting. This provided data on the evolution of CO_2 from degradation of NG with time.

RESULTS AND DISCUSSION

Anaerobic Studies:

Figure 1 shows the gas production with time for the mixture of NG (500 ppm), PGDN (500 ppm) and TMETN (500 ppm). It can be seen that the mixture degraded very easily and cumulative methane production corresponded to the data obtained for individual compounds.

Figure 2 shows the cumulative methane produced for TMETN water at a concentration of 1500 ppm. The cumulative gas production was higher than that for 1000 ppm indicating that at this concentration, TMETN had no toxicity effects to the culture. In the earlier study toxicity effects were found for TMETN water at 2000 ppm concentration. Hence it can be concluded that toxicity

effects begin to occur between 1500 ppm and 2000 ppm.

Aerobic Studies:

Figure 3 shows the oxygen uptake curve for 100 ppm NG water when mixed with 20 ppm each of PGDN and TMETN. In the previous study it was found that 100 ppm NG had degraded in about 100 hours. With 20 ppm each of PGDN and TMETN, the extent of NG degradation was decreased, as noted by the oxygen uptake curve. The total oxygen demand gave a plateau at 80 mg/L of oxygen, when the theoretical oxygen demand should have been 120 mg/L. This indicated that 20 ppm of PGDN and TMETN was toxic to the aerobic culture.

Radiolabelled NG concentration as measured by the scintillation counter showed that the disintegrations per minute (dpm) had decreased from an initial value of 2000 to 542 in the aqueous solution, indicating partial degradation of NG.

FUTURE WORK

The organisms isolated by Dr. Marilyn Speedie at the University of Maryland at Baltimore are currently being cultured. Aerobic studies will be conducted with these organisms.

FIGURE 1: CUMULATIVE METHANE PRODUCTION FOR A MIXTURE OF NG, PGDN, TMETN

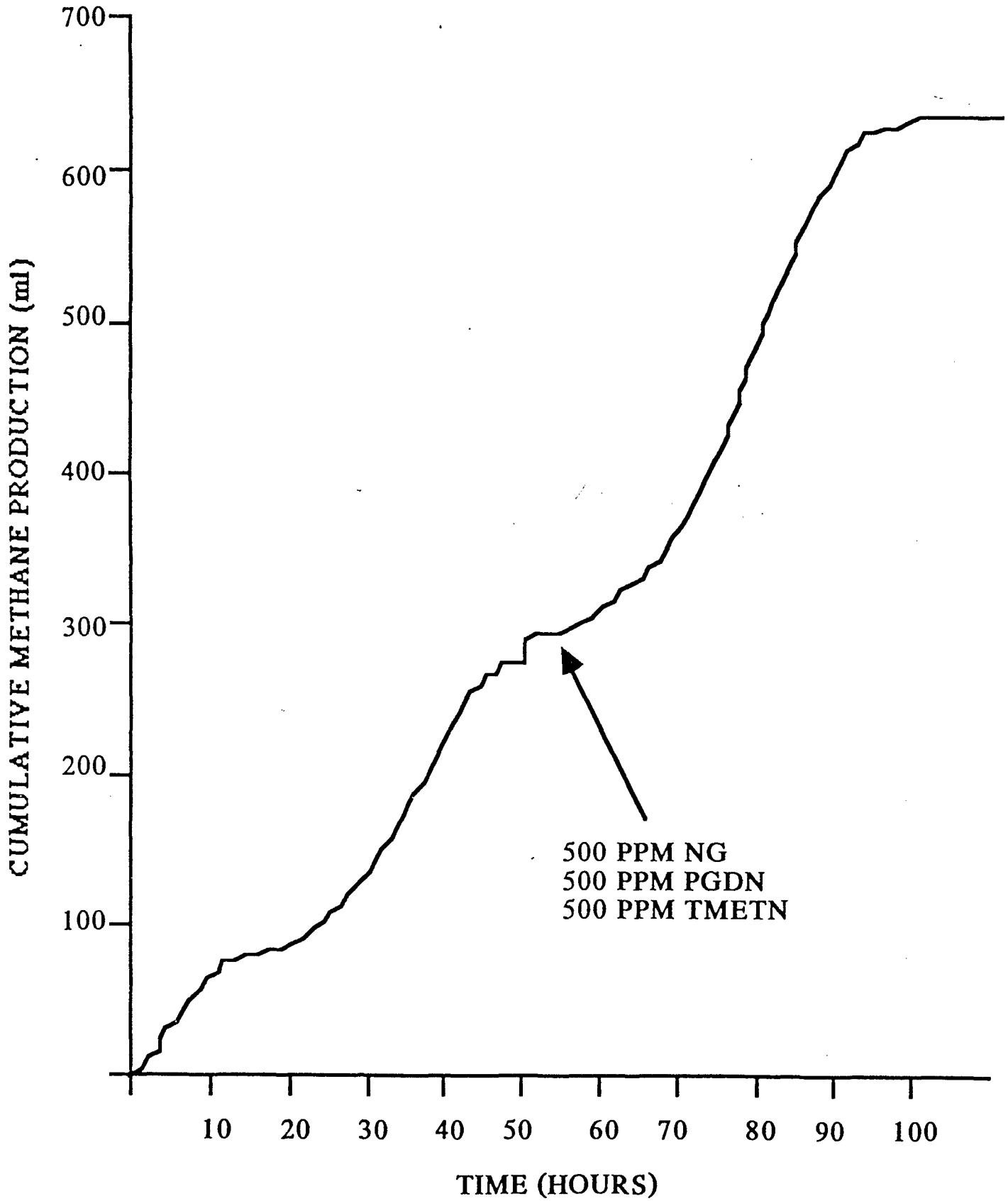


FIGURE 2: CUMULATIVE METHANE PRODUCTION FOR TMETN WATER

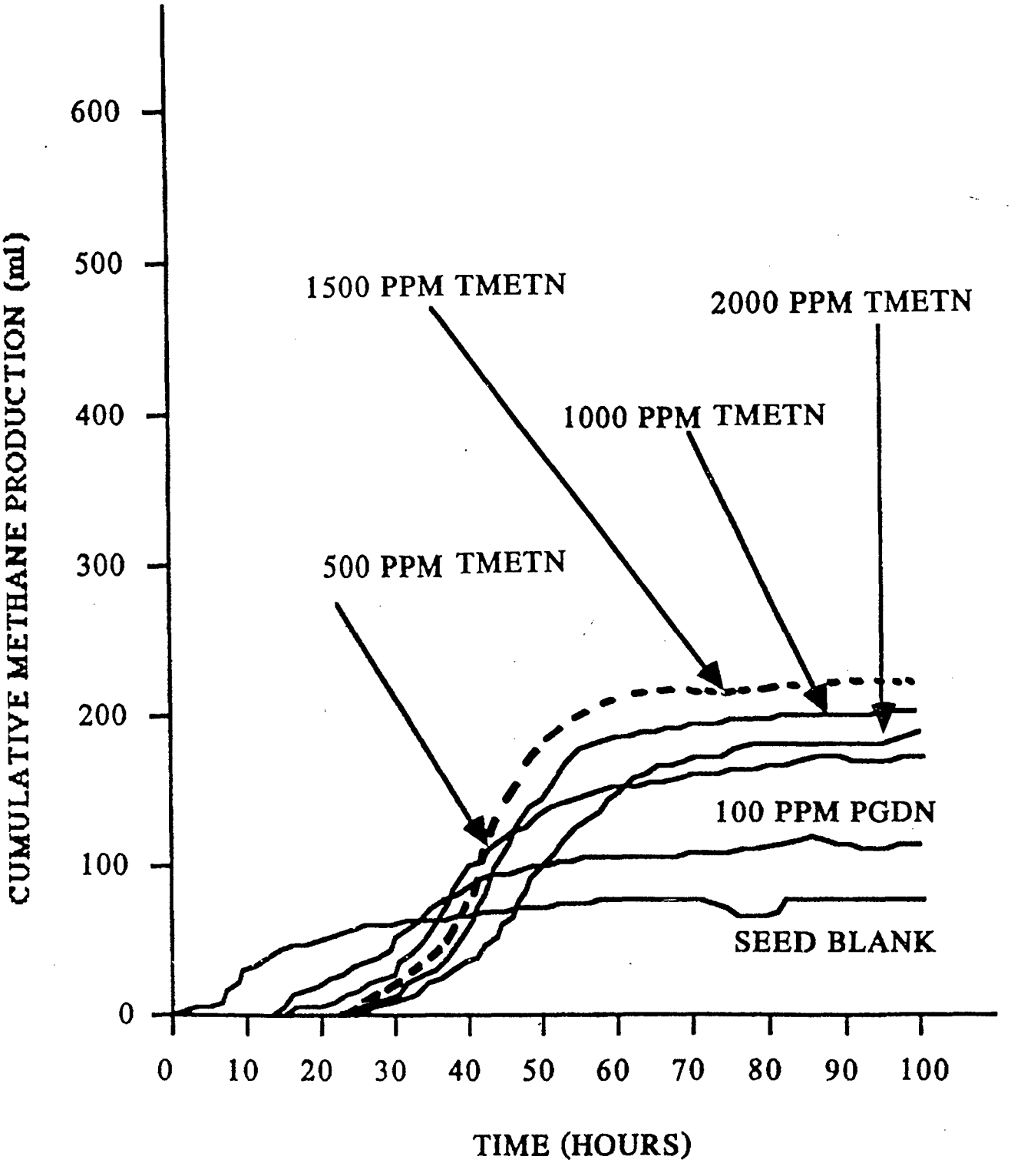
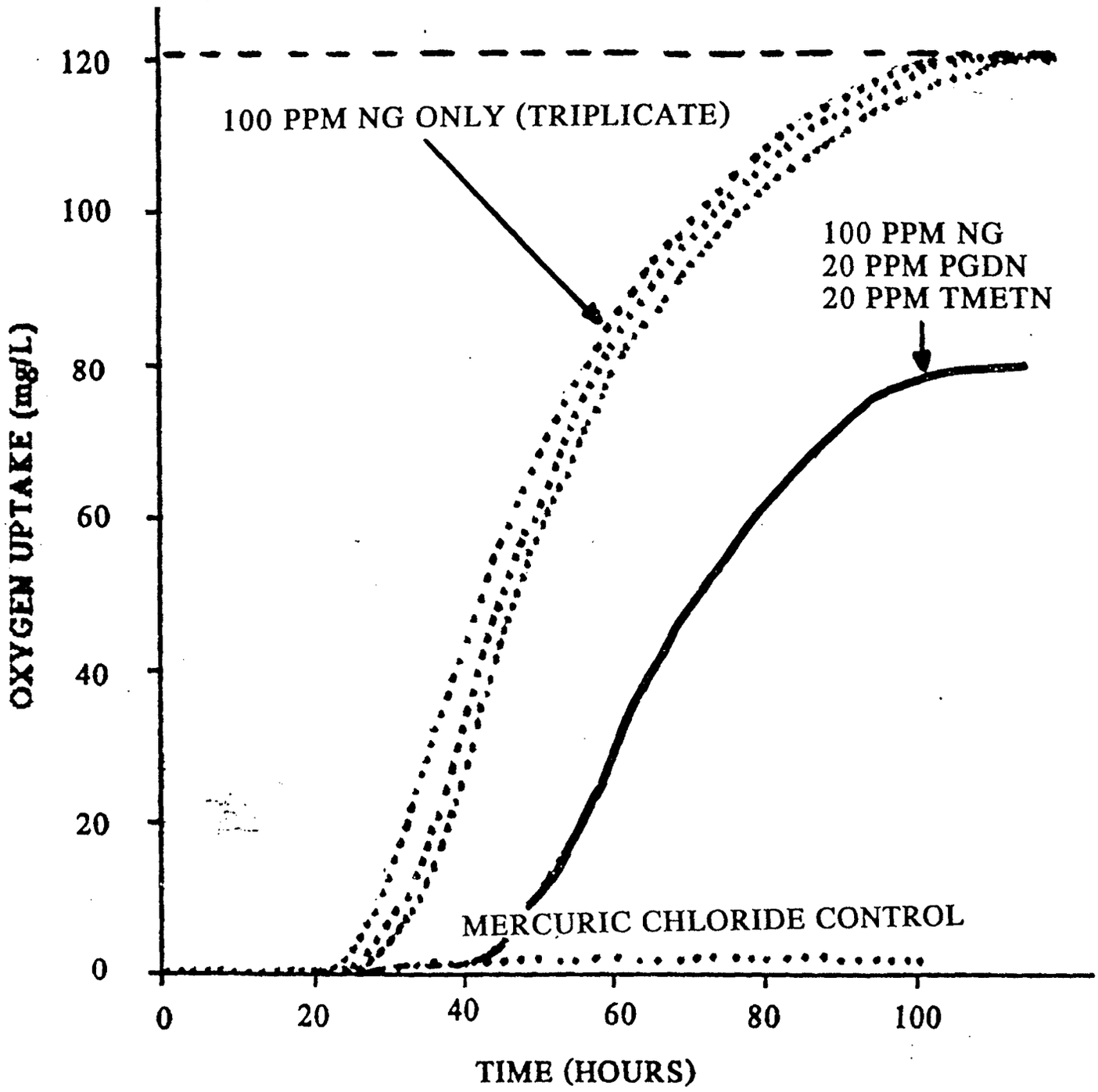


FIGURE 3: OXYGEN UPTAKE CURVE FOR AEROBIC DEGRADATION OF NITROGLYCERINE (NG).



FIRST QUARTERLY REPORT

on

STUDIES ON THE BIODEGRADATION OF ORDNANCE-RELATED HAZARDOUS WASTE

CATEGORY: MANUFACTURING OF ORDNANCE AND ORDNANCE COMPONENTS AND CHEMICALS

Submitted to: John Stacey
Code 2810C
Naval Ordance Station
Indian Head, MD 20640-5000

Submitted by: Dr. Rakesh Govind
Department of Chemical Engineering
Mail Location 171
University of Cincinnati
Cincinnati, OH 45221
Telephone: (513) 556-2666
Fax No. (513) 556-3473

MARCH 11, 1992

TABLE OF CONTENTS

Introduction 1

Project Objectives Addressed in this Report 3

Background 3

Experimental Studies 5

Development of acclimated master cultures 5

Use of Electrolytic Respirometry for Aerobic Kinetics 6

Determination of Anaerobic Kinetics 10

Preparation of chemical stock solution and analytical methods 11

Results and Discussion 11

Electrolytic Aerobic Respirometry Results 11

Anaerobic Serum Bottle Results 12

Future Work 13

References 14

Table 2

Figures

INTRODUCTION

The molecular processes involved in microbial and enzymatic degradation are being exploited by agencies of the U.S. Department of Defense in diverse ways. Biodegradation often provides an attractive or adjunct to conventional methods of restoring contaminated soils or groundwater, or treating wastewater containing hazardous chemicals. In this report, biodegradation refers to the use of microorganisms or enzymes to break down chemical compounds.

In 1980, Congress enacted legislation to identify, finance, and monitor cleanup of the nation's most serious hazardous waste dumps. Currently, DOD has 739 sites in need of remediation, and cleanup costs are estimated at five to 10 billion U.S. dollars. The primary hazardous wastes found on military installations are fuels, cleaning solvents, propellants, explosives, etc. Specifically, this proposal is addressing ordance-related hazardous chemicals, such as Nitroglycerine, Glycerol trinitrate, Propylene Glycol Dinitrate, Triethylene glycol dinitrate, and Tri methyl ethane trinitrate.

Virtually all conventional technologies for land reclamation have proved to be unsatisfactory. For example, landfilling is not a permanent solution, and costs are increasing dramatically (in the United States, from US $10 to US $250 per ton in the last five years). Incineration produces toxic air pollutants, and many organic compounds are difficult to burn. In situ solidification (chemical fixation), capping, and vitrification are largely

unproved, costly, and aesthetically objectionable technologies. Volatile organics in groundwater have been removed via strategically located slotted wells and by air stripping where contaminated groundwater is pumped through large packed-bed towers; both are costly, labor-intensive solutions and require further treatment of the contaminated air stream that is generated.

Furthermore, conventional methods for treatment of wastewater are expensive and require further treatment of the gas and solid phases that are generated. For example, conventional activated sludge treatment requires large-scale plant size, with a retention time in the range of 6-9 days, and generates contaminated air phase and sludges requiring further treatment.

Bioprocessing or biodegradation often provides a low-technology, permanent, inexpensive, effective, nonpolluting alternative for land reclamation and treatment of industrial effluents [1]. Microbes have evolved or can be adapted to degrade virtually any toxic organic chemical. Hydrocarbons, a major class of military wastes, are particularly susceptible to biodegradation. Biodegradation is effective over a range of environmental conditions, and for a wide variety of contaminants. Often, bioprocesses can be integrated with conventional technologies, resulting in efficient, multicomponent systems.

The overall goal of the proposed study is to address the following areas for the following chemicals: Nitroglycerine, Glycerol trinitrate, Propylene glycol dinitrate, Triethylene glycol dinitrate and Trimethyl ethane trinitrate:

1. Determining factors to optimize biodegradation under laboratory and field conditions;
2. Use of immobilized microbes and enzymes for biodegradation and development of field bioreactors; and
3. Developing new technologies for treating wastewaters, groundwater, air and soil contamination.

PROJECT OBJECTIVES ADDRESSED IN THIS REPORT

The specific objective of this interim report is to determine aerobic and anaerobic biodegradation kinetics for the following compounds: Nitroglycerine, Propylene Glycol dinitrate (PGDN), and Trimethyloethane trinitrate (TMETN) using electrolytic respirometry (aerobic) and biological methane production (anaerobic).

BACKGROUND

The microbial degradation of glycerol trinitrate (GTN) has been reported in the literature [2]. The degradation process proceeds through a series of successive denitration steps through glycerol dinitrate and glycerol mononitrate isomers, with each succeeding step proceeding at a slower rate.

In addition to direct microbial degradation, chemical treatments of GTN have been developed in order to desensitize waste streams, resulting in the disappearance of glycerol tri-, di-, and mononitrates, but with the corresponding formation of glycidol and glycidyl nitrate [3]. These products contain a highly reactive epoxide moiety that tends to confer mutagenic properties.

The chemical by-products, glycidol and glycidyl nitrate, have also been studied to determine their biodegradability. The pathway from glycidyl nitrate to glycerol 1-mononitrate to glycerol proceeds more slowly with each succeeding step. The steps from glycidol to glycerol and glycidyl nitrate to glycerol 1-mononitrate occur spontaneously in aqueous solutions but appear to be accelerated (directly or indirectly through secondary effects) by microbial activity [3].

Glycidol and glycidyl nitrate tested positive in the Ames test screening for mutagenicity, whereas the transformation product glycerol-1-mononitrate tested negative [3]. Both glycerol trinitrate and glycerol-1-mononitrate have been shown to be toxic to mammals [4].

Propylene glycol dinitrate (PGDN), diehylene glycol dinitrate (DEGN), triethylene glycol dinitrate (TEGDN), and trimethyllolethane trinitrate (TMETN) undergo microbial transformation via successive denitration steps, leading to the formation of the corresponding glycols:propylene glycol (PG), diethylene glycol (DEG), triethylene glycol (TEG), and trimethylolethane glycol (TMEG) (refer Figure 3)[5].

The degradation of the resulting glycols at concentrations of 100 mg per liter has also been assessed [6]. Rates of degradation were as follows: PG > DEG > TEG > TMEG, from high to low, although degradation appeared to be due to a combination of biological and non-biological processes.

PG, DEG, and TEG present minimal toxicity and carcinogenic

hazards. PG is the least toxic of the glycols and is commonly used in pharmaceutical, cosmetic, and food applications. DEG and TEG are slightly toxic; repeated large doses are needed for toxicity [7]. TMEG had negative test results in AMES test screening for mutagenicity [6].

A generic biological treatment concept for wastewaters contaminated with hazardous Nitrate Esters is as follows:

```
INFLUENT  ----> CHEMICAL PRETREATMENT ------>
                  (if required)

ANAEROBIC
DENITRIFICATION ------> AEROBIC TREATMENT ---> EFFLUENT
```

Mixed cultures were used in many of the studies reported here. The ability of microorganisms to biotransform this class of compounds is apparently ubiquitous in nature. The use of acclimatized cultures from environments previously exposed to the compounds under study perhaps would have accelerated some of the initial rates of transformation observed; however, once acclimatized, random environmental microbial inocula were capable of transforming the compounds studied.

EXPERIMENTAL STUDIES

Development of acclimated master cultures:

Master cultures were developed for three organic compounds: Propylene Glycol Dinitrate, Trimethyloethane Trinitrate, and Nitroglycerine, by exposing wastewaters containing these compounds

to a domestic wastewater treatment plant culture. A batch stirred tank reactor, 5 liters in volume, was operated at 10 C to prevent growth of nitrifying bacteria, with continuous aeration of air. To start the reactor, activated sludge and sewage from the Miami Municipal Treatment Plant in Cincinnati, Ohio, was centrifuged and then allowed to settle in settlers. The supernatant was filtered through a #1 Whatman filter to remove protozoa, and then filled into the batch stirred tank master culture reactor. Wastewaters, supplied by the Navy, were diluted with distilled water (1:8 ratio), and then supplied to the master culture reactor. The feed to the reactor (200 ml fed daily) consisted of an equimolar mixture of PGDN, TMETN, and GTN diluted wastewaters. 200 ml of OECD composition nutrients were also fed daily. 400 ml of mixed liquor was withdrawn daily from the reactor, to maintain the volume of liquid in the reactor.

The reactor was monitored daily for nitrates and nitrites.

Use of Electrolytic Respirometry for Aerobic Kinetics:

Electrolytic Respirometry studies were conducted using an automated continuous oxygen measuring Voith Sapromat B-12 (Voith-Morden, Milwaukee, WI). The system consists of a temperature controlled bath which contains the measurin units, a recorder for digital indication, a plotter for direct presentation of the oxygen uptake curves of substrates, and a cooling unit for conditioning and continuous recirculation of water bath contents. The system used has 12 measuring units each connected to a recorder. Each unit, consists of a reaction vessel, with a carbon dioxide absorber

(soda lime) mounted in a stopper, an oxygen generator, and a pressure indicator. The unit is sealed from the atmosphere, and hance fluctuations in baromatric pressure does not affect the results. The reactor contents are well stirred by a manetic stir bar, thus ensuring effective gas exchange. Biodegradation of the organic contents in the reactor vessel, creates carbon dioxide, which is absorbed by the soda lime pellets, thereby creating a negative pressure in the flask. This decrease in reactor vessel pressure trigers the pressure sensor which switches the oxygen generator. Oxygen is generated by electrolysis of a copper sulfate -sulfuric acid solution. The oxygen flows into the reactor vessel, thereby restoring the oxygen content in the headspace. The amount of oxygen generated is monitored by measurement of the voltage supplied to the electrolytic unit, and the cumulative oxygen generated per unit volume of the reactor flask contents, is plotted as the oxygen uptake curve.

The nutrient solution is made as per Organization of Economic Cooperation and Development (OECD) guidelines. It contains 10 ml of solution A and 1 ml of each of the solutions B to F per liter of synthetic medium : solution A - KH_2PO_4 8.5 g, K_2HPO_4 21.75 g, $Na_2HPO_4.2H_2O$ 33.4 g, and NH_4Cl 2.5 g; solution B - $MgSO_4.7H_2O$ 22.5 g; solution C - $CaCl_2$ 27.5 g; solution D - $FeCl_3.6H_2O$ 0.25 g; solution E - $MnSO_4.4H_2O$ 39.9 mg, H_3BO_3 57.2 mg, $ZnSO_4.7H_2O$ 42.8 mg, $(NH_4)_6Mo_7O_{24}$ 34.7 mg and $FeCl_3$.EDTA 100 mg; and solution F - Yeast extract 150 mg. The chemicals for each of the solutions A, B, C, D, E and F, are dissolved in 1000 ml of deionized water. The solution D is

freshly prepared immediately before the start of an experiment.

The microbial inoculum was obtained from the acclimated culture reactor by centrifuging the mixed liquor. The dry weight of biomass was measured by drying samples, in duplicates, of 1 ml, 2 ml, and 3 ml at 105 C overnight. A concentration of 30 mg/l of sludge as dry weight was used in each reactor vessel. The total volume of the synthetic medium in the 500 ml capacity reactor vessels was brought up to a final volume of 250 ml.

The concentration of the test compound was varied. Aniline was used as the biodegradable reference compound. The design of the experimental runs is shown in Table 1.

Table 1: Experimental Design of the Aerobic Respirometer Runs

RESPIROMETER NUMBER __

CHEMICAL NAME: _____

Flask No.	Inoculum	Chemical	Nutrient	Water
1	8 ml	25 ml	167 ml	50 ml
2	8 ml	25 ml	167 ml	50
3	8 ml	25 ml	167 ml	50
4	8 ml	50 ml	167 ml	25
5	8	50	167	25
6	8	50	167	25
7	8	75	167	0
8	8	75	167	0
9	8	75	167	0
10	8	25+5A	167	0
11	8	25+5A	167	0
12	8+2 ml $HgCl_2$	50	167	2 3

Note that the chemical was added in amounts of 25 ml, 50 ml and 75 ml of 1000 ppm stock solution. The nutrients was basic OECD synthetic medium. Deionized distilled water was used to make up the flask contents to 250 ml. In flasks 10 and duplicate 11, a solution of aniline (5 gm/l concentration), which is the reference compound, was used as a toxicity control. In flask 12, 2 ml of a mercuric chloride (200 ppm concentration) was added, so that no bacterial action occurs.

Determination of Anaerobic Kinetics:

A standard batch anaerobic bioassay technique (Biological Methane Potential (BMP) and Anaerobic Toxicity Assay (ATA)) was used to determine the methane potential and the anaerobic toxicity of Nitroglycerine, TMETN, and PGDN.

The BMP assay was conducted with Corning no. 1460, 250 ml reagent bottle and ATA assay was conducted with 125 ml reagent bottles with rubber septum caps of appropriate size. The bottles were gassed at a flow rate of approximately 0.5 l/min. with 30% CO2 and 70% N2, then stoppered and equilibrated at incubation temperature of 35 C prior to introducin samples, defined media and inoculum. The defined media contained nutrients and vitamins for mixed anaerobic cultures. Resazurin was added to detect oxygen contamination and sodium sulfate was added to provide a reducing environment. The final assay concentrations of nitrogen, phosphorus, and alkalinity were respectively: 12 mg/l as N, 19 mg/l as P and 2500 mg/l as CaCO3.

For BMP assay, inoculation was accomplished anaerobically by inserting a gas flushing needle into the neck of the media flask while adding 200 ml of seed organisms to 1800 ml of defined media. A 20% by volume inoculum was used.

Samples were added to the serum bottles anaerobically, and experiments at concentrations of 100 ppm, 500 ppm, 1000 ppm, 2000 ppm were run in triplicate. A seed blank with no addition of chemical was also run in triplicate.

For ATA assay, a "spike" containing acetate and propionate in

addition to the seed blanks was also added to each bottle. A control with only the spike was also prepared. Each bottle contained 2 ml of acetate-propionate solution containing 75 mg acetate and 26.5 mg popionate.

Gas volume production was measured with the help of a calibrated pressure transducer. The composition of the gas was also measured using a Gas Chromatograph.

Preparation of Chemical Stock Solution and analytical methods:

The water sample, sent by the Navy, was extracted with ethyl ether in a continuous extractor for 24 hours, which very efficiently extracted essentially all the nitrate ester compounds. The extracts were concentrated by evaporation of solvent, at room temperature. Quantitative analysis of the nitrate ester compound was made by using a Hewlett Packard GC/MS and a Varian 4100 HPLC in conjunction with a silica column (Varian Micropak Si, 10 um) 50 cm in length with 2.2 mm ID. Analysis was conducted at 220 nm with a back pressure of 1,800 lb/sq. in.

The analyses were calibrated using the standards, sent by the Navy.

RESULTS AND DISCUSSION

Electrolytic Aerobic Respirometry Results

The oxygen uptake results are shown in Figures 1 through 3 for Nitroglycerine, Propylene Glycol Dinitrate and Trimethyloethane Trinitrate compounds, respectively. Note that Propylene glycol Dinitrate (PGDN) and Trimethyloethane Trinitrate (TMETN) were not

biodegraded at all in the respirometer. Nitroglycerine was degraded in the respirometer, but there was a long acclimation period of 24 hours (refer Figure 1) before glycerol was formed. The glycerol product was easily biodegraded. Aniline degradtion in the presence of nitroglycerine (Figure 2) showed delayed onset of aniline degradation and incomplete mineralization of the aniline. In Figure 2, the first plateau of oxygen uptake is attained due to aniline degradation, followed by degradation of nitroglycerine.

Figure 3 shows the oxygen uptake curve for PGDN solutions. Note that no substantial uptake of oxygen occurred, even when aniline was present in the solution. This shows that PGDN was not degraded aerobically and that it also inhibited the degradation of aniline. Similar results were obtained for TMETN, as shown in Figure 4.

No attempts were made to quantify the aerobic biodegradation kinetics, since the compounds were either very slow to acclimate or did not biodegrade at all, at the three concentrations, used in the respirometer. Clearly, aerobic biodegradation conditions were not optimal for biotransformation of these compounds.

Anaerobic Serum Bottle Results

The anaerobic serum bottle results (BMP) are shown in Figures 5 through 7, for Nitroglycerine, PGDN and TMETN, respectively. Each curve was obtained as the average of the triplicate runs. The concentration of the compounds were 100 ppm, 500 ppm, 1000 ppm, and 2000 ppm.

All three compounds degraded anaerobically at all

concentrations. Nitroglycerine (Figure 5) degraded very rapidly and showed no toxicity to the anaerobes. PGDN (Figure 6) degraded slower than nitroglycerine, and note that the gas production at 2000 ppm was lower than at 1000 ppm, indicating some toxicity to the anaerobic culture. TMETN (Figure 7) degradation kinetics was slower than PGDN and also exhibited toxicity to the culture at 2000 ppm.

Figure 8 shows the ATP test consisting of nitrolycerine water and a spike of acetate and propionate. The methane production curve for the control flask, containing acetate and propionate only, has been plotted in all subsequent figures. Figure 9 shows the ATP test with PGDN water. Again note that the methane produced from the 2000 ppm flask is less than that produced by the 1000 ppm flask. Figure 10 shows the ATP test for TMETN water.

Clearly, anaerobic degradation would be very successful in degrading these chemical compounds. However, it should be mentioned, that maintenance of anaerobic conditions, in actual bioreactors is difficult, and would require very careful design for successful operation.

Attempts to obtain the upper limiting concentration for PGDN and TMETN when toxicity is created for the anaerobic culture were unsuccessful, due to gas volume measurement errors. These runs are being repeated now, and results of these studies will be reported in the next interim report.

The BMP results were analyzed using a standard Haldane kinetics model, and the resulting kinetic constants are given in

Table 2. These values of the kinetic constants would be useful in the design of the biofilter system for biological treatment of wastewaters containing these compounds.

FUTURE WORK

Studies are being planned to study the degradation kinetics of these compounds under facultative conditions, wherein the electron acceptor is either sulfate or nitrate. Studies are also planned with radiolabelld compounds, for mixtures of these compounds, using scintillation counting techniques. Uniformly labelled nitroglycerine has been synthesized and currently the safety protocol for radiolabelled compounds is being developed.

REFERENCES

[1] K. Gold and B.W. Brodman. U.S. Patent No. 4,756832 (july 12, 1988).

[2] T.M. Wendt, J.H. Cornell, and A.M. Kaplan, Applied Environ. Microbiol., 36, 693 (1978).

[3] D.L. Kaplan, J.H. Cornell, and A.M. Kaplan, Applied Environ. Microbiol., 43 (1), 144 (1982).

[4] L.V. Melinikoveend, A.M. Klyachkina, Toksikol Nov. Prom. Khima Veshchestv, 15, 97 (1979).

[5] J.H. Cornell et al., Report No. 81.029, U.S. Army Natick Research, Development and Engineering Center, Natick, MA 1981.

[6] D.L. Kaplan, J.T. Walsh, and A.M. Kaplan, Environ. SCi. Tech. 16 (10), 723 (1982).

[7] R.E. Gosselin et al., Chemical Toxicity of Commercial Products, 4th Edition (Williams and Wilhem, Baltimore, MD 1976).

Table 2: Summary of the kinetic constants for anaerobic degradation of Nitroglycerine, PGDN and TMETN

Toxicant	Concentration (ppm)	k (mg/mg VSS-hr)	Ks (mg/l)
Nitro-Glycerine	100	0.26	45
	500	0.25	48
	1000	0.27	47
	2000	0.26	50
PGDN	100	0.15	82
	500	0.14	85
	1000	0.16	80
	2000	0.08	95
TMETN	100	0.10	95
	500	0.09	100
	1000	0.08	102
	2000	0.04	110

FIGURE 1: Oxygen Uptake curves for aerobic degradation of Nitroglycerine (Flasks 1,2,3 in Table 1). The lowest curve is the mercuric chloride control.

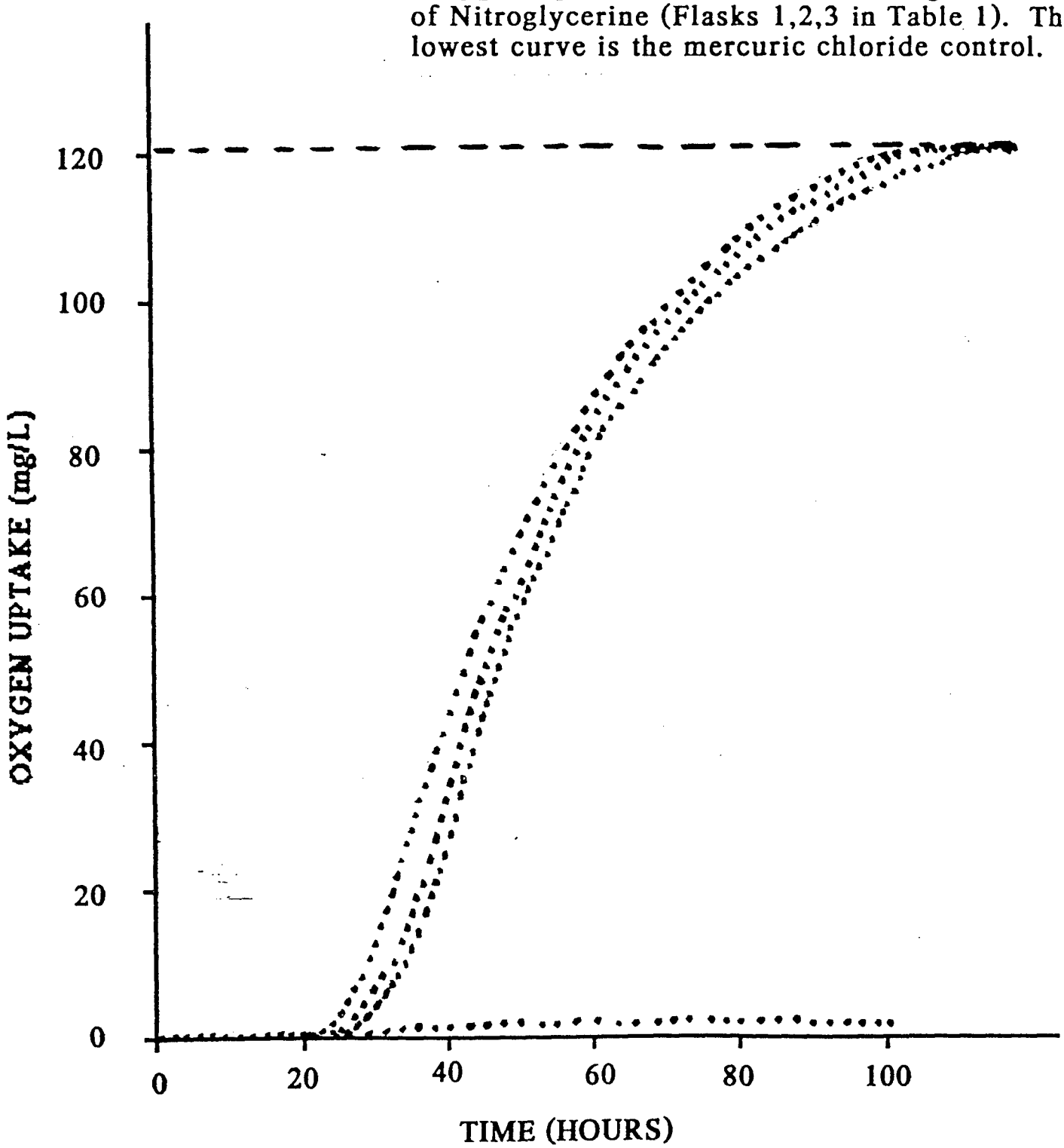


FIGURE 2: Oxygen Uptake curves for aerobic degradation of Nitroglycerine with Aniline (Flask 10 in Table 1). The lowest curve is the mercuric chloride control.

Note that aniline degradation was delayed, due to toxicity effects. Also the aniline did not degrade completely.

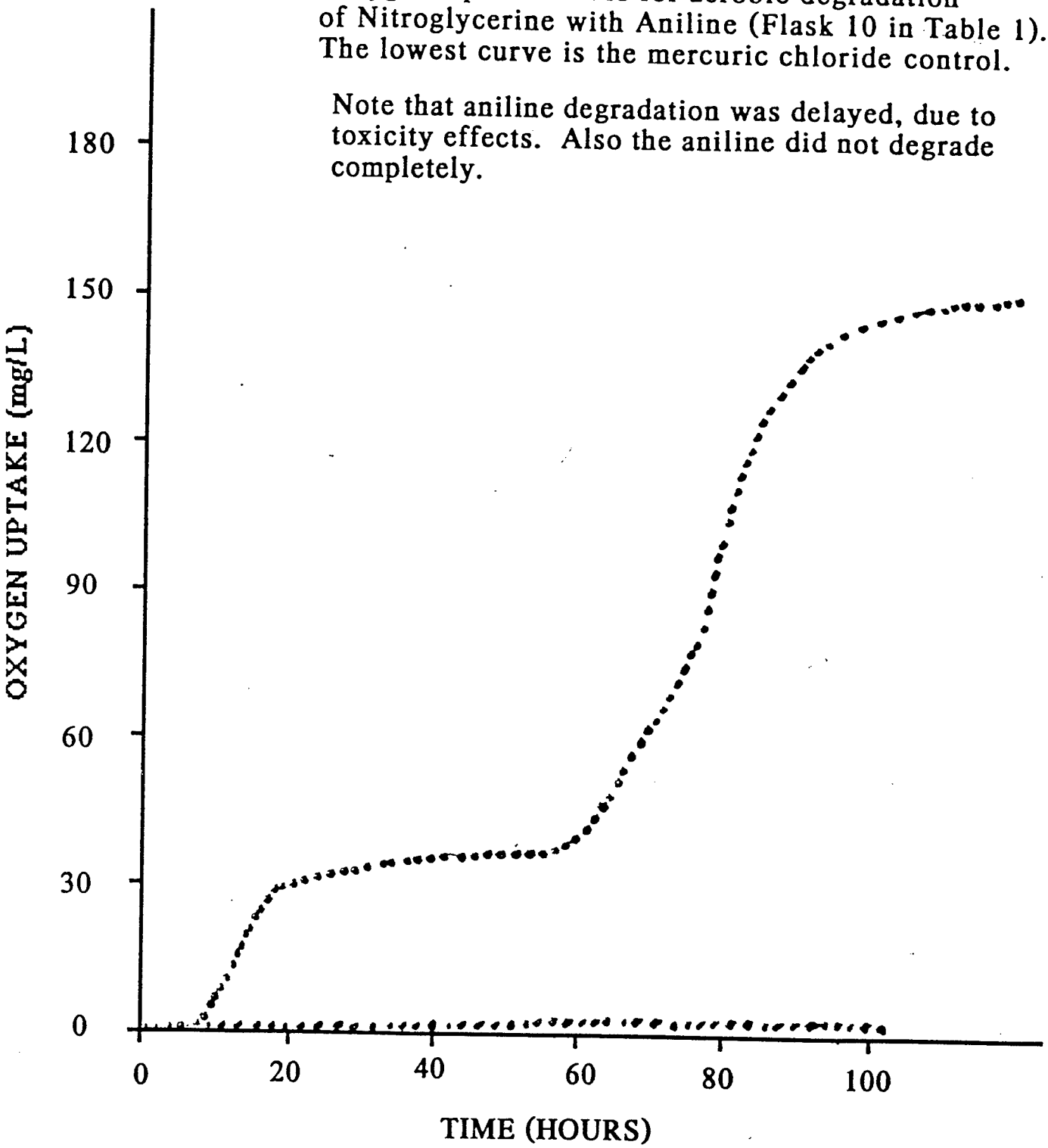


FIGURE 3: Oxygen uptake curve for PGDN solution. Note that oxygen uptake was very small for all concentrations of PGDN. The three curves represent the average of flasks (1,2,3) (4,5,6) and (7,8,9) [Refer Table 1]. No oxygen uptake was measured for flasks 10 &11. Hence, no aniline degradation occured due to toxicity of PGDN.

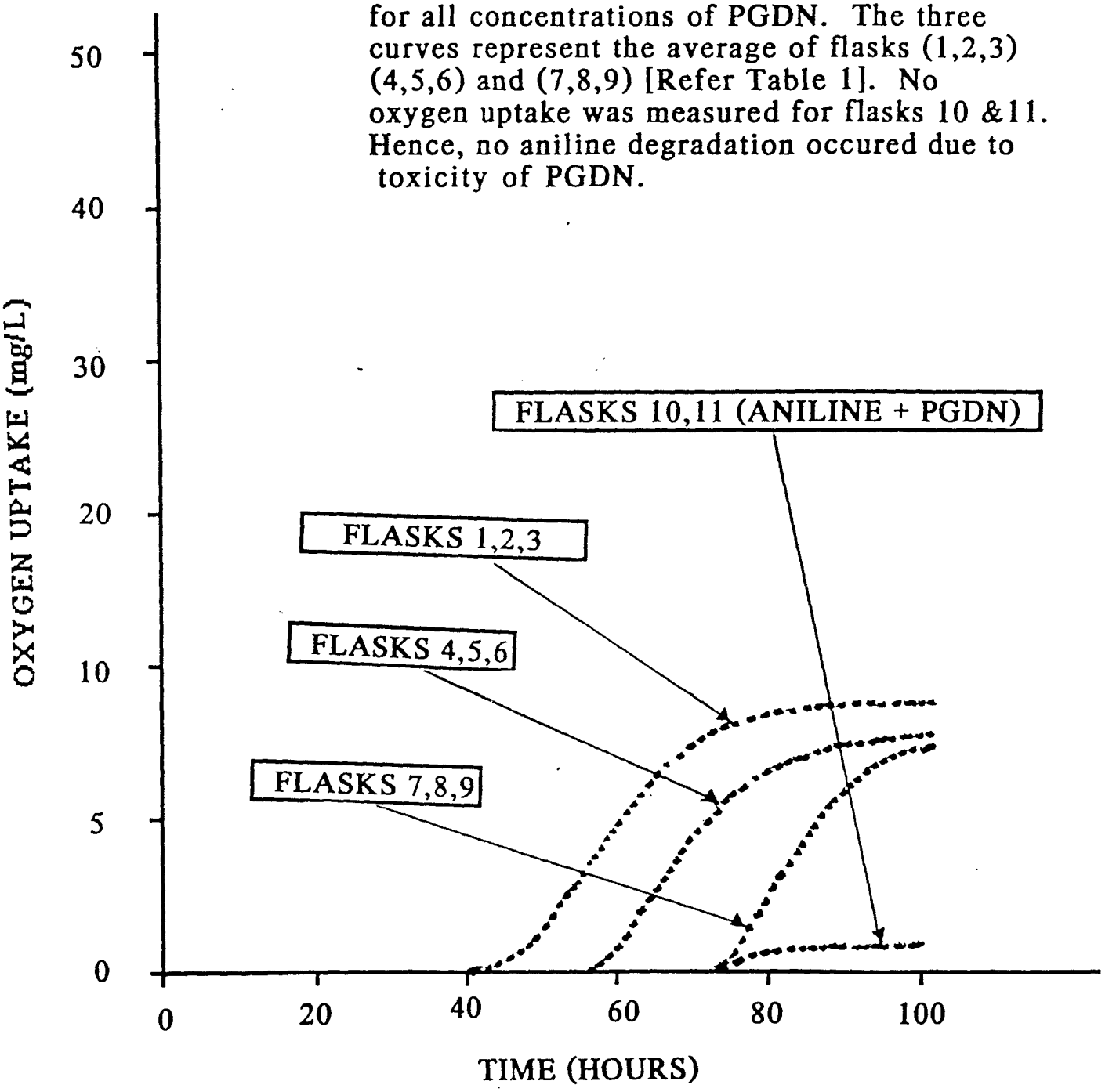


FIGURE 4: Oxygen uptake curve for TMETN solution. Note that oxygen uptake was very small for all concentrations of TMETN. The three curves represent the average of flasks (1,2,3) (4,5,6) and (7,8,9) [Refer Table 1]. No oxygen uptake was measured for flasks 10 &11. Hence, no aniline degradation occured due to toxicity of TMETN.

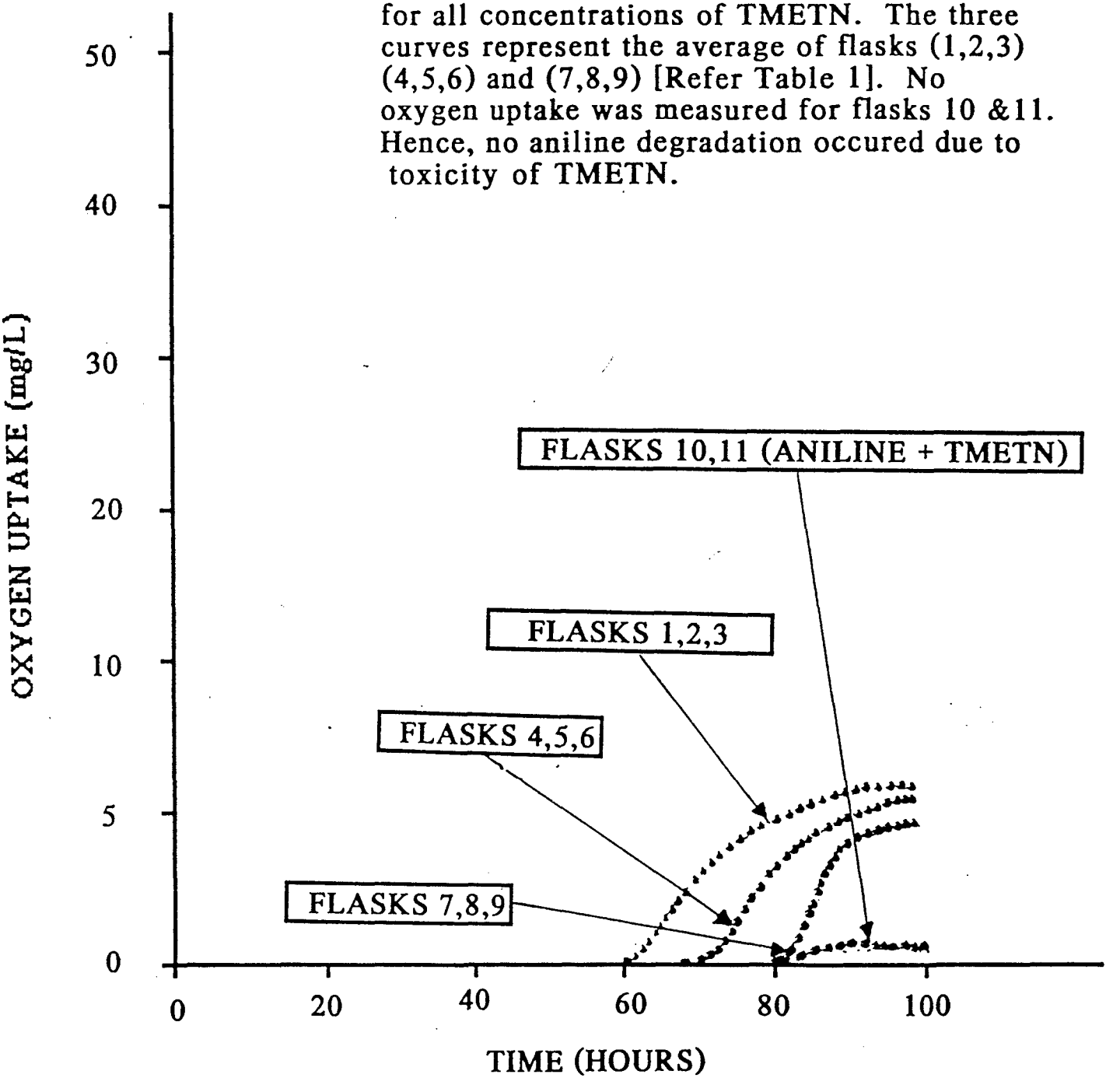


FIGURE 5: CUMULATIVE METHANE PRODUCTION FOR NITROGLYCERINE WATER

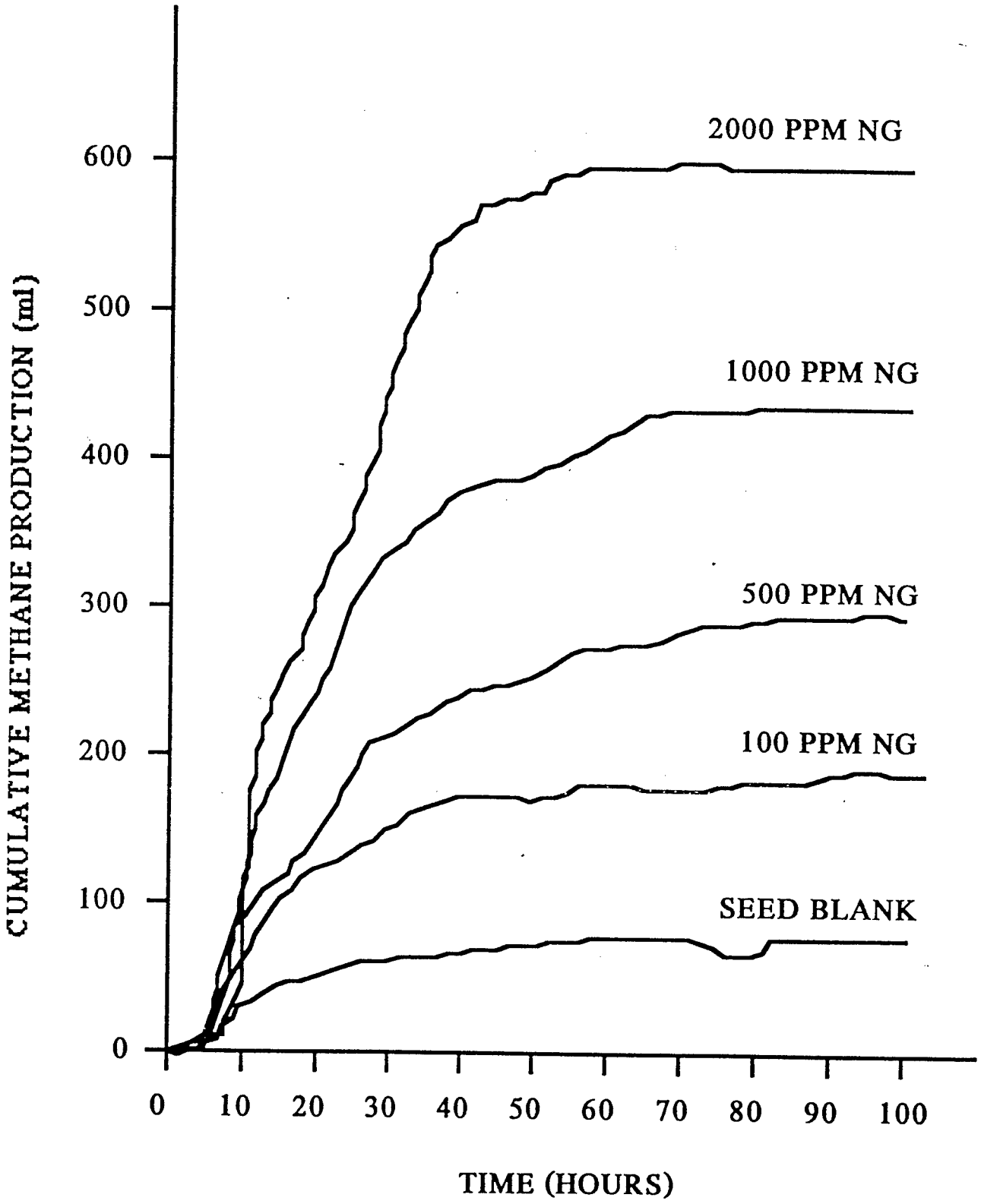


FIGURE 6: CUMULATIVE METHANE PRODUCTION FOR PGDN WATER

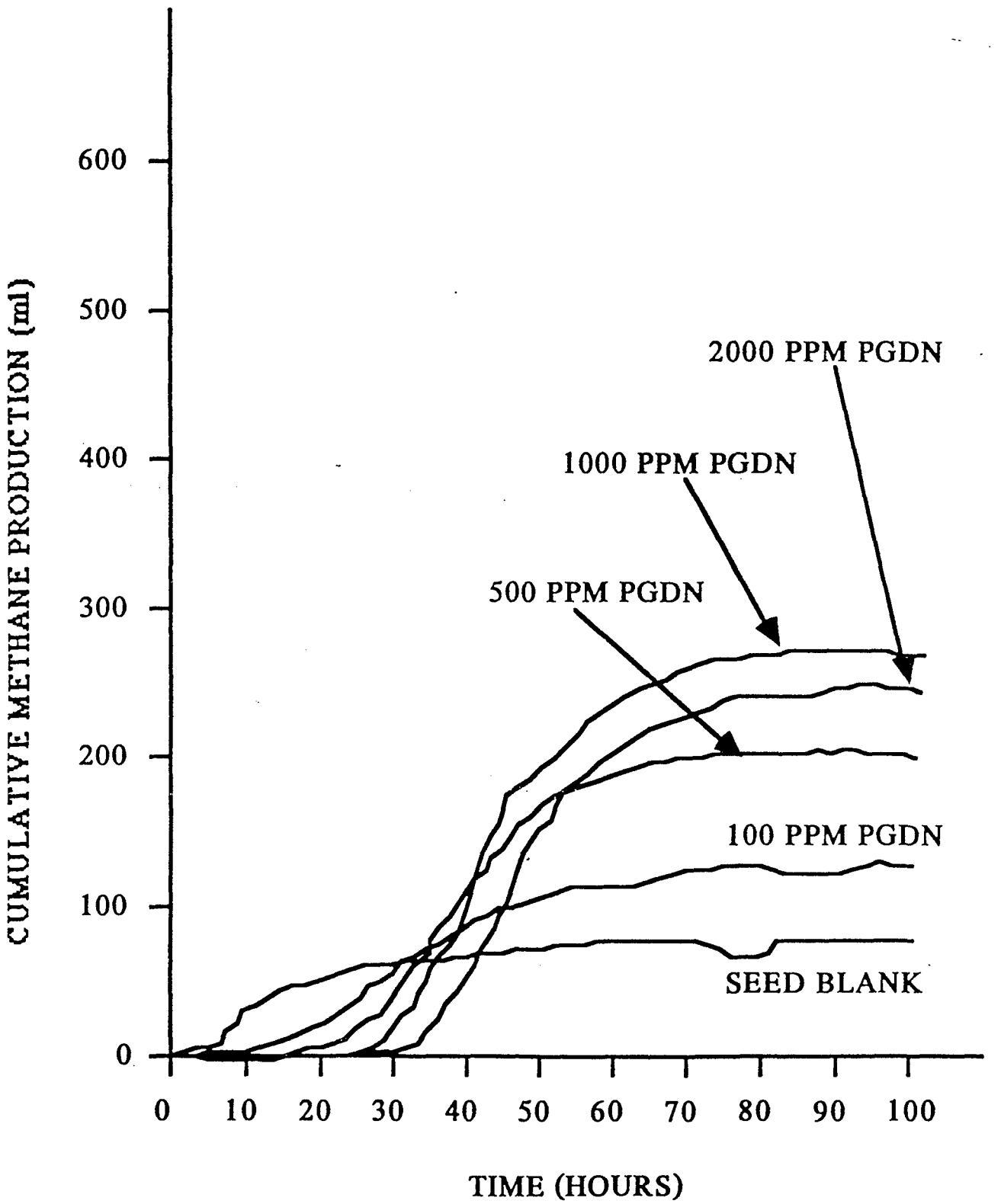


FIGURE 7: CUMULATIVE METHANE PRODUCTION FOR TMETN WATER

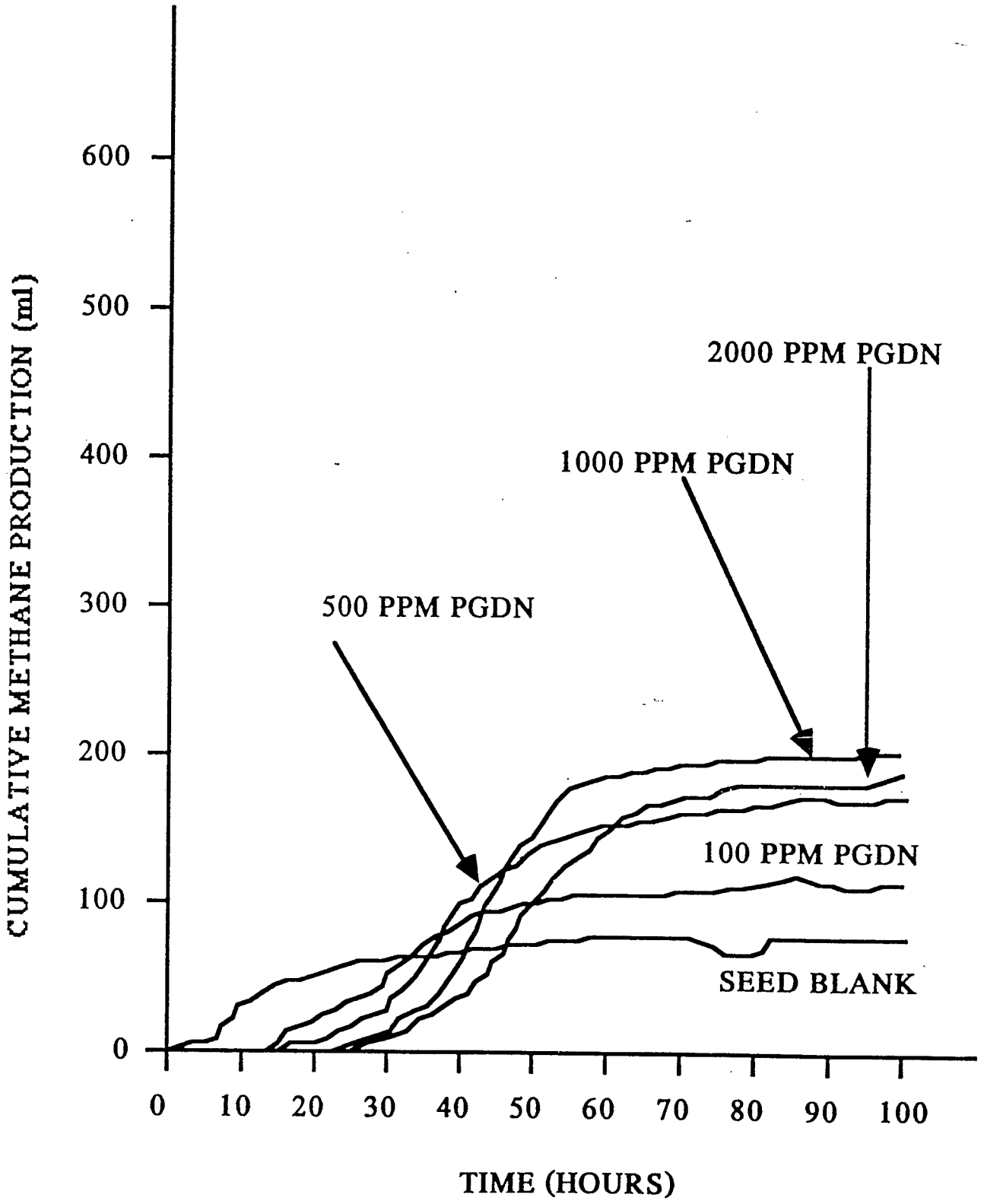


FIGURE 8: CUMULATIVE METHANE PRODUCTION FOR NITROGLYCERINE WATER WITH SPIKE OF ACETATE AND PROPIONATE

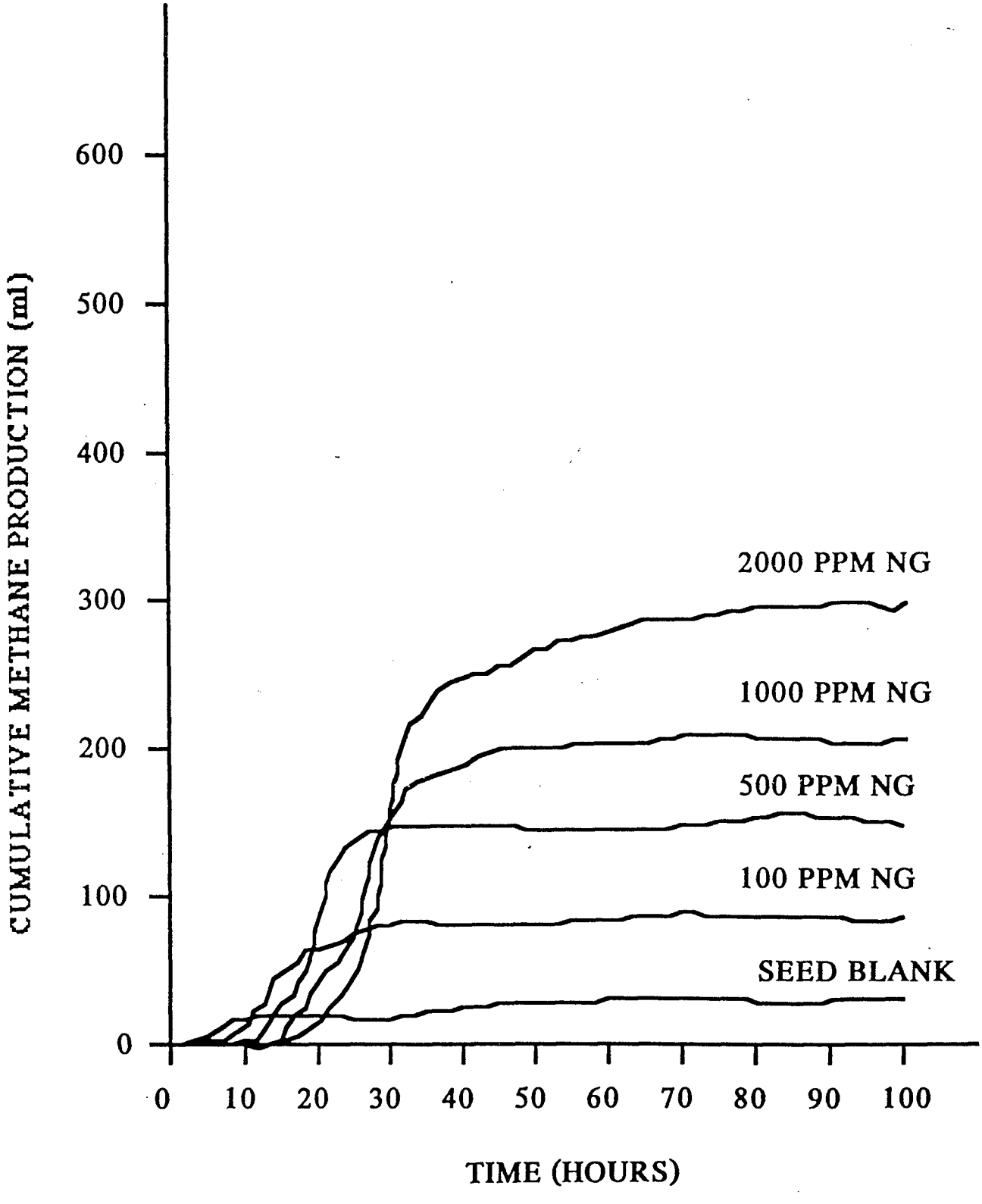


FIGURE 9: CUMULATIVE METHANE PRODUCTION FOR PGDN WATER WITH ACETATE AND PROPIONATE SPIKE

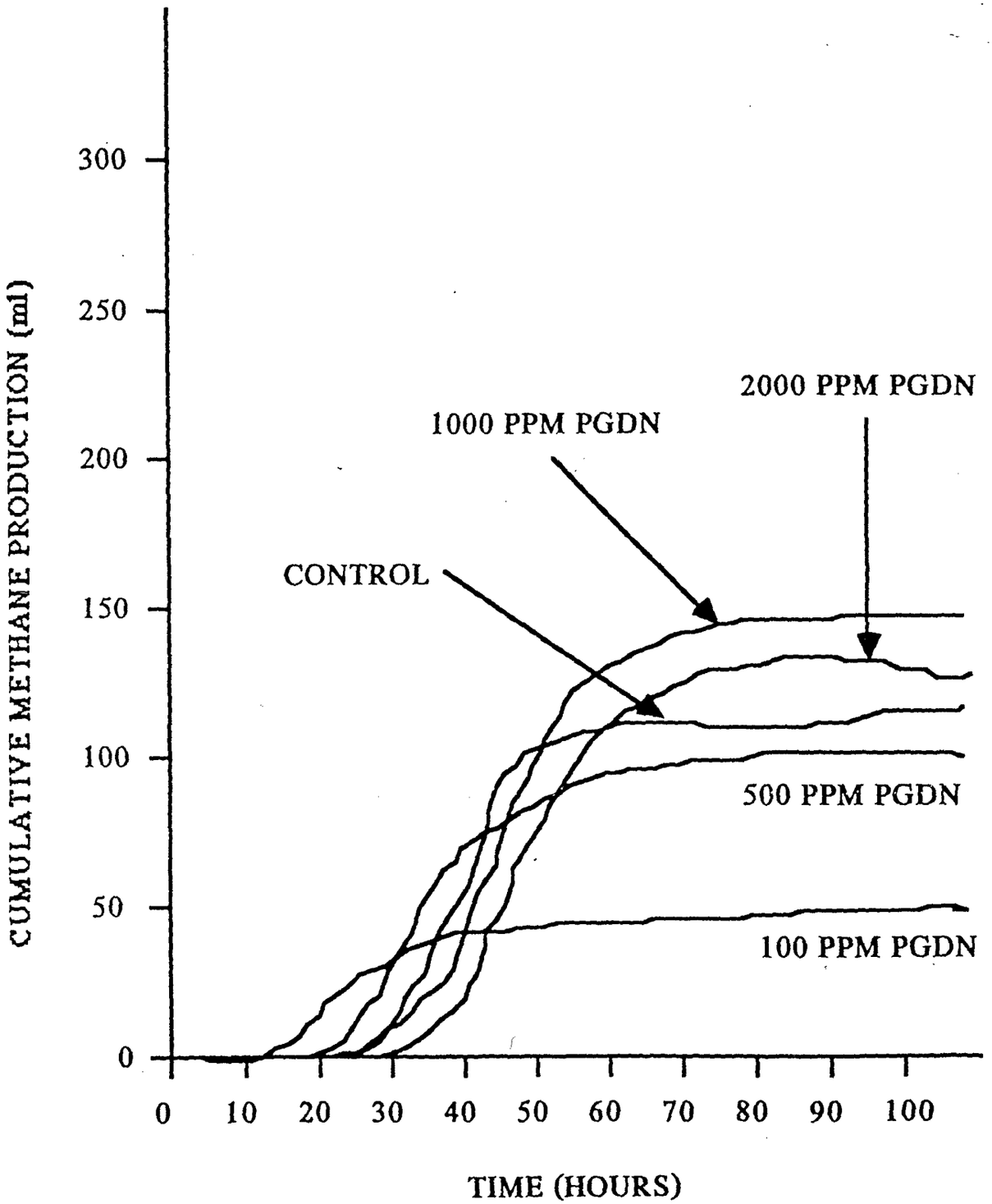


FIGURE 10: CUMULATIVE METHANE PRODUCTION FOR TMETN WATER WITH ACETATE AND PROPIONATE SPIKE